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Characterization of soyasaponin metabolism by human gut microorganisms and bioavailability in humans

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Characterization of soyasaponin metabolism by human gut microorganisms and bioavailability in humans

by

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A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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For the Major Program

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ABSTRACT

Soyasaponins have demonstrated health-promoting properties including plasma cholesterol-lowering, anti-carcinogenic and hepato-protective. Significant amounts of soyasaponins are found in soybeans and soy products. The role of soyasaponins in promoting improved health status has led to a need in understanding their bioavailability and metabolism in humans.

Metabolism of soyasaponin I (3-*O*-[α -*L*-rhamnopyranosyl- β -*D*-galactopyranosyl- β -*D*-glucuronopyranosyl]-olean-12-en-3 β ,22 β ,24-triol) by human gut microflora was investigated to elucidate the metabolism of dietary soyasaponins in human intestine. In a static *in vitro* fecal fermentation model, disappearance of soyasaponin I displayed an apparent first-order kinetics over 48 h. Two soyasaponin degradation phenotypes were observed among the subjects: rapid degraders with $k = 0.23 \pm 0.04 \text{ h}^{-1}$, and slow with $k = 0.07 \pm 0.02 \text{ h}^{-1}$. Two primary gut metabolites of soyasaponin I were identified as soyasaponin III (3-*O*-[β -*D*-galactopyranosyl- β -*D*-glucuronopyranosyl]-olean-12-en-3 β ,22 β ,24-triol) and soyasapogenol B (4-methoxyl-olean-12-en-3 β ,22 β ,24-triol).

Bioavailability of dietary soyasaponins was assessed in a human feeding study. After a single oral dose of soy drink, no soyasaponins or soyasapogenols was detected in the 24 h urine. About 8.6% of ingested group B soyasaponins was recovered as the form of soyasapogenol B, a major gut metabolite of group B soyasaponins, over a 5-day feces collection, suggesting dietary soyasaponins could be metabolized to soyasapogenols by gut microflora *in vivo* and excreted in feces.

The cellular absorbability and transport kinetics of soyasaponins was evaluated using Caco-2 transfer model, a human colon carcinoma cell model. The apical-to-basolateral absorption of soyasaponin I and soyasapogenol B was low with P_{app} of 0.9 to 3.5×10^{-6} cm/sec and 0.3 to 0.6×10^{-6} cm/sec, respectively. Caco-2 cells were able to uptake soyasaponin I and soyasapogenol B from the apical membrane. The accumulation of soyasaponin I in Caco-2 cells displayed a saturable and concentration-independent kinetics, while soyasapogenol B accumulated in Caco-2 cells in a concentration dependent manner. Soyasaponin I was not cytotoxic to Caco-2 cells at ≤ 3 mM, while soyasapogenol B at ≥ 1 mM significantly decreased cell viability in the culture.

These findings suggest that ingested soyasaponins can be metabolized by human gut microorganisms to smaller and more hydrophobic molecules. Individuals may vary in their ability to metabolize soyasaponins in the gut. Dietary soyasaponins and its gut metabolite soyasapogenols may have very low absorbability in the human intestine.

GENERAL INTRODUCTION

A. Introduction

Saponins are the triterpenoid or steroid glycosides naturally occurring in plants and some marine organisms. Relatively high concentrations of saponins have been found in soybeans and soy products. The primary saponins in soybeans are the bisdesmosidic group A soyasaponins and the monodesmosidic group B soyasaponins. Saponins in the whole soybean seeds constitute about 60 to 75% group B soyasaponins and 25 to 40% group A soyasaponins by weight (Ireland *et al.* 1986; Gu *et al.* 2002). The dry weight basis concentration of soyasaponins has been reported to range from 1.4 to 5.9 $\mu\text{mol/g}$ in soybeans, 0.2 to 114 $\mu\text{mol/g}$ in soy ingredients, and 1.5 to 4.5 $\mu\text{mol/g}$ in soy foods such as tofu, miso, soymilk and tempeh (Kitagawa *et al.* 1984; Ireland *et al.* 1986; Tsukamoto *et al.* 1995; Hu *et al.* 2002; Gu *et al.* 2002).

Soyasaponins have been proposed to have certain health protective activities associated with soy consumption. Soyasaponins have been broadly believed as the major active components contributing to the cholesterol-lowering effect of soy products (Potter *et al.* 1995; Okenfull 2001). Soyasaponins inhibited various types of tumor development *in vivo* and *in vitro*, particularly colon cancer development (Rao *et al.* 1995; Koratkar *et al.* 1997). Group B soyasaponins appeared to be inhibitory to human immunodeficiency virus (HIV) replication and infection *in vitro* (Hayashi *et al.* 1996). Hepato-protective activity of soyasaponins has been observed in a number of *in vitro* studies (Kim *et al.* 1997; Miyao *et al.* 1997). Soyasaponins have displayed the ability to protect fibroblast cells from oxidative damage (Yoshikoshi *et al.* 1996). However, many of the studies investigating soyasaponin

biological activities have been limited to *in vitro* experiments and a few animal studies. The relevance of these findings to humans under *in vivo* conditions is not clear.

Little is known about the bioavailability and metabolism of soyasaponins in animals and humans in spite of their health-promoting potential. There is also no direct *in vivo* evidence for many of the biological activities associated with orally ingested saponins. Saponins have been assumed to be poorly absorbed in the intestine. It was believed that their sugar chains had to be hydrolyzed to liberate aglycones by bacterial enzymes in the lower intestine (Gestetner *et al.* 1968; Karikura *et al.* 1990). There is no information on the fate of soyasaponins in the human gut, and also no data on their absorption and pharmacokinetics in animals or humans.

B. Objective of current research

The role of dietary soyasaponins in the potential promotion of improved health status, especially in cholesterol lowering and cancer inhibition, leads to a need to understand their bioavailability in humans. The overall objective of my doctorate research was to elucidate the metabolism of purified soyasaponins in the human intestine by gut microorganisms, and evaluate bioavailability of dietary soyasaponins in humans. Although it was unknown whether and how soyasaponins were metabolized by human gut microflora, we hypothesized that the sugar moiety of soyasaponins could be hydrolyzed in a stepwise manner to produce a series of secondary metabolites and eventually liberate the aglycones; and that the hydrophobic metabolites might be absorbed in the intestine.

To test the hypothesis, three specific aims of my study were proposed: 1. to investigate the metabolism of soyasaponin I and its catabolic pattern by human gut

microorganisms and to identify the possible major metabolites using an *in vitro* static fermentation model; 2. to examine the individual variability of soyasaponin metabolism among human subjects and explore the factors affecting gut microbial degradation of soyasaponins; 3. to evaluate absorbability of dietary soyasaponins and their possible microbial metabolites in humans through a human feeding experiment and an *in vitro* Caco-2 cell transport assay.

The information obtained in this study will help to predict metabolism and bioavailability of dietary soyasaponins in the human intestine and to gain a better understanding on the potential and mechanisms of health-promoting properties of soyasaponins.

C. Dissertation organization

This dissertation consists of a literature review and two papers. The first paper, "Metabolism of soyasaponin I by human intestinal microflora", will be submitted to the Journal of Agriculture and Food Chemistry. The second paper, "Human intestinal absorption and bioavailability of soyasaponin I", will be submitted to the Journal of Nutrition. The papers are written in the format of the journals to which they will be submitted. A general conclusion will be included following the two papers.

LITERATURE REVIEW

A. Soyasaponins

Saponins are a family of steroid or triterpenoid glycosides present in plants. More than a thousand different types of saponins have been identified in a wide variety of plants. The basic structure of saponins is a triterpenoid or steroid aglycone attached to one or more sugar chains, resulting in an amphiphilic nature of the molecules (Hostettmann and Marston 1995).

Soyasaponins are oleanene-type triterpenoid saponins. Soyasaponins can be divided into two groups, A and B, according to their respective aglycones, soyasapogenol A and soyasapogenol B (Figure 1). The group A soyasaponins are bisdesmoside saponins with two different polysaccharides attached to C-3 and C-22 positions of soyasapogenol A (Figure 2). Eight isomers of group A saponins, named Aa, Ab, Ac, Ad, Ae, Af, Ag, and Ah according to their elution order in reverse phase high performance liquid chromatography (HPLC),

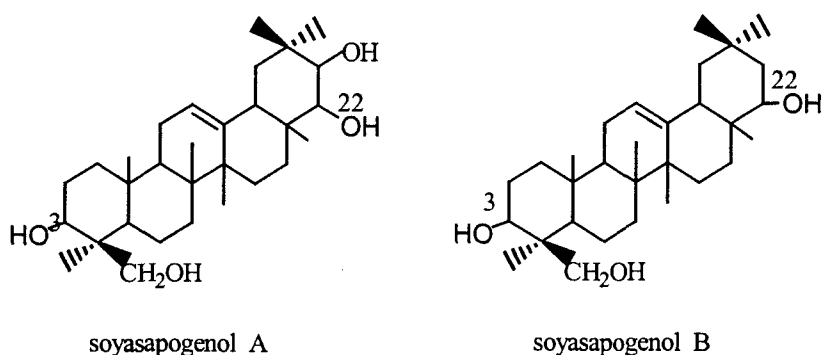
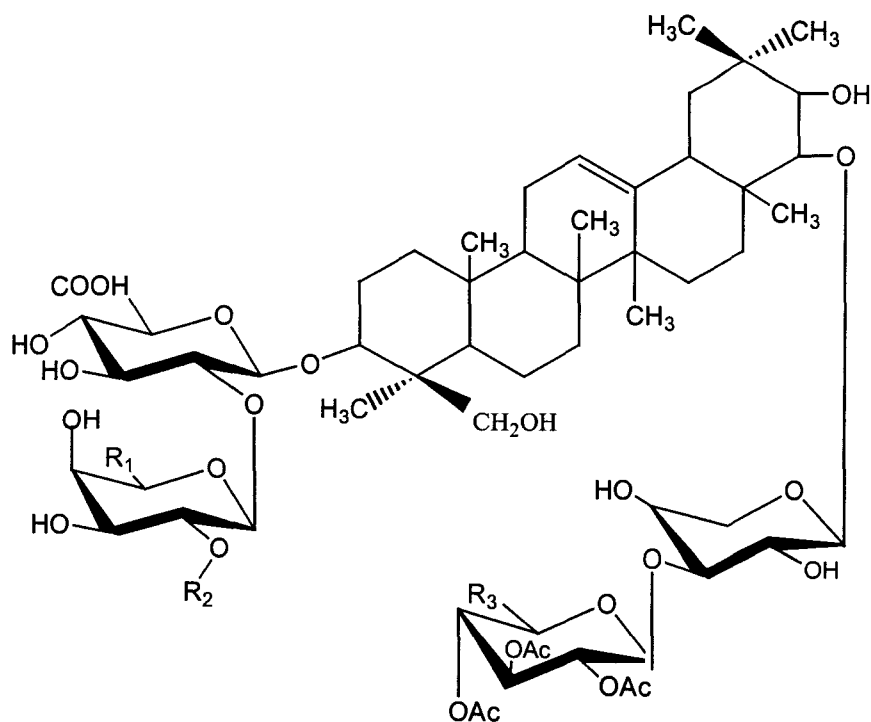


Figure 1. Structures of soyasapogenols

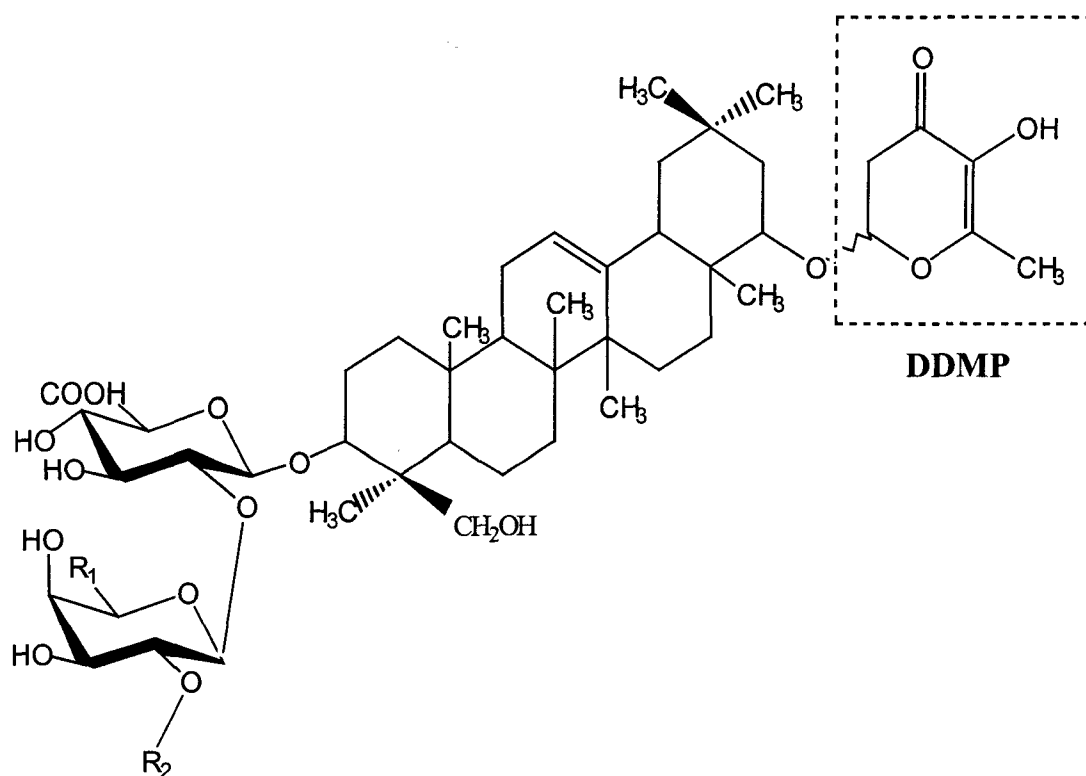
were isolated from soybeans and characterized by Shiraiwa *et al.* (1991). These group A soyasaponins have a terminal xylose or glucose residue attached to C-22. Hosny and Rosazza (2002) recently isolated and identified two new group A soyasaponins from soybeans, which were characterized as 3-O- $\{[\alpha\text{-L-rhamnopyranosyl-(1}\rightarrow\text{2)}\text{-}\beta\text{-D-galactopyranosyl-(1}\rightarrow\text{2)}\text{-}\beta\text{-D-glucunonopyranosyl}]\text{-22-O-}[\alpha\text{-L-rhamnopyranosyl-(1}\rightarrow\text{2)}\text{-}\alpha\text{-L-arabinopyranosyl}]\}$ -3 β ,22 β ,24-trihydroxyl-olean-12-ene, and 3-O- $\{[\alpha\text{-L-rhamnopyranosyl-(1}\rightarrow\text{2)}\text{-}\beta\text{-D-galactopyranosyl-(1}\rightarrow\text{2)}\text{-}\beta\text{-glucunonopyranosyl}]\text{-22-O-}[\alpha\text{-L-rhamnopyranosyl-(1}\rightarrow\text{2)}\text{-}\beta\text{-glucopyranosyl}]\}$ -3 β ,22 β ,24-trihydroxyl-olean-12-ene. The two new group A soyasaponins have a terminal rhamnose residue instead of a xylose or glucose residue.

The group B soyasaponins are monodesmoside saponins with one di- or tri-saccharide chain attached to soyasapogenol B. The group B soyasaponins isolated from soybeans have a 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP) group conjugated to C-22. Five isomers of group B soyasaponins, named soyasaponin βg , βa , γa , γg and αg , have been isolated from soybeans (Figure 3) (Kudou *et al.* 1993). Several authors reported that DDMP conjugated soyasaponins were not stable and were easily converted into non-DDMP soyasaponins, named soyasaponin I, II, III, IV and V, respectively, during heated extraction procedures or cooking, in the presence of Fe, in alkaline solution, or upon storage in alcoholic solution at room temperature for several days (Massiot *et al.* 1996; Daveby *et al.* 1998; Yoshiki *et al.* 1998; Gu *et al.* 2002).



	R1	R2	R3
Soyasaponin Aa	CH ₂ OH	β-D-Glc	H
Soyasaponin Ab	CH ₂ OH	β-D-Glc	CH ₂ OAc
Soyasaponin Ac	CH ₂ OH	α-L-Rha	CH ₂ OAc
Soyasaponin Ad	H	β-D-Glc	CH ₂ OAc
Soyasaponin Ae	CH ₂ OH	H	H
Soyasaponin Af	CH ₂ OH	H	CH ₂ OAc
Soyasaponin Ag	H	H	H
Soyasaponin Ah	H	H	CH ₂ OAc

Figure 2. Structures of group A soyasaponins (Yoshiki *et al.* 1998)
 Glc: glucopyranosyl; Rha: rhamnopyranosyl; Ac: acetyl



	R1	R2	DDMP
Soy saponin βg	CH ₂ OH	α-L-Rha	Y
Soy saponin I	CH ₂ OH	α-L-Rha	N
Soy saponin βa	H	α-L-Rha	Y
Soy saponin II	H	α-L-Rha	N
Soy saponin γg	CH ₂ OH	H	Y
Soy saponin III	CH ₂ OH	H	N
Soy saponin βa	H	H	Y
Soy saponin IV	H	H	N
Soy saponin αg	CH ₂ OH	β-D-Glc	Y
Soy saponin V	CH ₂ OH	β-D-Glc	N

Figure 3. Structures of group B soyasaponins (Kudou *et al.* 1994)

Rha: rhamnopyranosyl; **Glc:** glucopyranosyl;

DDMP: 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one

Y: yes; **N:** no.

B. Food sources and dietary intake of soyasaponins

Soyasaponins have been found in variety of leguminous plants such as mung beans, cowpeas, scarlet runner beans, lentils, chickpeas, kidney beans, lupine seeds and alfalfa (Price *et al.* 1986; Tsukamoto *et al.* 1994; Kinjo *et al.* 1994; Oleszek *et al.* 1998). The reported concentration of soyasaponins in the leguminous seeds are summarized in Table 1. Genuine DDMP-conjugated group B soyasaponins have been found in various plants including *Pisum sativum* (Tsurumi *et al.* 1992), *Phaseolus coccineus* (Yoshiki *et al.* 1994), *Lupinus angustifolius* (Ruiz *et al.* 1995), and *Medicago sativa* (Massiot *et al.* 1992). Okubo's group (1996) determined the composition of DDMP-conjugated soyasaponins in forty-one varieties of leguminous seeds. They found that soyasaponin β g appeared to be the most prevalent form of soyasaponins and was present in thirty varieties among the legume seeds they analyzed.

Table 1. Soyasaponin contents in various leguminous seeds

Variety	Soyasaponin contents (g/100g)	Reference
Kidney beans	total saponins, 0.35	Price <i>et al.</i> 1986
Runner beans	total saponins, 0.34	Price <i>et al.</i> 1986
Soybeans	DDMP-conjugated saponins, 0.22-0.24	Tsukamoto <i>et al.</i> 1995
Cowpeas	soyasaponin I and V, 0.12	Kinjo <i>et al.</i> 1998
Garden peas	soyasaponin I, 0.04-0.06	Kinjo <i>et al.</i> 1998
Peanuts	soyasaponin I, 0.10	Kinjo <i>et al.</i> 1998
Broad beans	soyasaponin I, 0.05	Kinjo <i>et al.</i> 1998
Chickpeas	soyasaponin I and β g, 0.071-0.075	Ruiz <i>et al.</i> 1996
Lentils	soyasaponin I and β g, 0.09-0.11	Ruiz <i>et al.</i> 1996, 1997

Soybean seeds contain about 2% glycosides mainly in the forms of isoflavones and soyasaponins (Tsukamoto *et al.* 1995). Whole soybean seeds contain about 60 to 75 % group B soyasaponins and 25 to 40 % group A soyasaponins according to the soyasaponin profiles reported by Ireland *et al.* (1986) and Gu *et al.* (2002). Group A soyasaponins are distributed mainly in the hypocotyls of soybean seeds (Shiraiwa *et al.* 1991). Group B soyasaponins in the soybean seeds are located in the plumule, hypocotyl and radicle (Tani *et al.* 1985). The concentration of soyasaponins in the soybean seeds varies in different genetic background and growth stages of soybeans as well as cultivation environment (Shimoyamada *et al.* 1991; Tsukamoto *et al.* 1995; Hu *et al.* 2002).

Soybeans and soy foods are the major source of dietary soyasaponins consumed by humans. Soyasaponin contents in some soy ingredients and soy foods reported in the literature are summarized in Table 2. The composition and concentration of soyasaponin isomers in soy products are quite different depending on the product types and processing conditions. Hu *et al.* (2002) evaluated group B soyasaponin contents in various soy foods and soy ingredients. We found that DDMP-conjugated soyasaponins were the primary group B soyasaponins detected in the raw soybean flour at a level of 3.3 $\mu\text{mole/g}$, whereas non-DDMP soyasaponins were the major forms detected in the processed soy products and ingredients with concentrations ranging from 0.2 to 114 $\mu\text{mole/g}$. The traditional soy foods such as soymilk, tempeh and tofu, appear to be low in soyasaponins compared to raw soybeans on an “as is” weight basis. However, soyasaponin concentrations on a dry weight basis in these soy foods were comparable to that in the raw soybean flour. Group B soyasaponins were undetectable in ethanol-washed soy protein concentrates but were high in

Table 2. Soyasaponin content and composition in commercial soy products *

	Group B soyasaponin content (μmol/g) **						
	V	I	II	αg	βg	βa	total
Soybean flour ¹	0.00	0.28	0.21	0.17	2.19	0.47	3.31
Tofu ²	0.00	0.31	0.13	0.01	0.11	0.03	0.59
Tempeh ³	0.00	0.76	0.39	0.01	0.28	0.09	1.53
Soymilk ⁴	0.00	0.22	0.12	0.00	0.09	0.04	0.47
Acid-washed soy concentrates ⁵	0.00	2.41	1.05	0.19	4.90	0.86	9.41
Ethanol-washed soy concentrates ⁵	0.00	0.08	0.12	0.00	0.00	0.00	0.20
Isolated soy protein 500E ⁶	0.87	5.73	2.39	0.10	1.20	0.31	10.60
Isolated soy protein Supro 670 ⁶	0.00	5.59	2.50	0.07	1.01	0.33	9.51
Textured vegetable protein ⁵	0.00	1.89	0.87	0.11	1.26	0.38	4.51
Soy hypocotyl ⁷	4.41	5.80	0.00	4.71	12.53	0.00	27.46
Novasoy® ⁵	0.00	77.55	36.48	0.00	0.00	0.00	114.02

	Group A soyasaponin content (μmol/g) ***								
	Aa	Ab	Ac	Ad	Ae	Af	Ag	Ah	
Soy germ ⁸	5.16	4.65	1.08	0.00	1.80	1.14	0.17	0.08	23.89
Soy cotyledon ⁸	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Defatted soy meal ⁹	0.00	0.74	0.25	0.19	0.00	0.30	0.00	0.07	1.55
Molasses ¹⁰	8.38	8.14	1.22	0.00	2.71	1.02	0.24	0.00	21.71

* All the samples were reported on as is weight basis

** Hu *et al.* J. Agri. & Food Chem. 2002; 50:2587-2594

*** Gu *et al.* J. Agri. & Food Chem. 2002; 50:6951-6959

¹ Vinton81, 1994 crop

² Mori-nu, firm

³ Quong Hop & Company

⁴ White Wave, Inc.

⁵ Archer Daniels Midland Company

⁶ Protein Technologies International

⁷ Schouten USA Inc., toasted

⁸ Kennong16, 1997 crop

⁹ Jilin3, 1997 crop

¹⁰ Shanghai Liantang Food Factory

acid-washed soy protein concentrates, which were similar to those of soy protein isolates. Group A soyasaponins appeared to be concentrated in soy hypocotyl and soy molasses with concentrations of 23.9 and 21.7 $\mu\text{mole/g}$, respectively, but very low in defatted soy meal as 1.6 $\mu\text{moles/g}$ (Gu *et al.* 2002). The concentrations of soyasaponins in soy protein isolates were significantly higher than in raw soybean flour. Soy hypocotyls are rich in both group A and B soyasaponins with reported concentrations of 23.9 and 27.5 $\mu\text{mole/g}$, respectively (Hu *et al.* 2002; Gu *et al.* 2002). Novasoy® is produced by drum-drying the alcoholic extracts from soy protein concentrate production and was commercialized by Archer Daniels Midland Company (ADM). Soyasaponin concentration is thirty-fold higher in Novasoy® compared to raw soy flour. Soy hypocotyls and Novasoy® are the major sources of various isoflavone dietary supplements in the market place. Thus, these isoflavone-rich supplements also provide enriched sources of soyasaponins.

Soy ingredients, such as soy protein isolate, soy protein concentrate and textured vegetable protein, have been used to produce the second generation soy foods because of their food processing functionalities as well as health promoting properties. The most widely available products are soy infant formulas, soy hot dogs, soy burgers, soy bacon and other soy-meat analogues. These products are low in soyasaponin content. Murphy's group (unpublished data 2002) reported 0.02 to 0.31 $\mu\text{mole/g}$ group B soyasaponins in meatless franks, 0.53 to 1.83 $\mu\text{mole/g}$ in Harvest burgers®, and undetectable in soy-beef patty. Interestingly, soy infant formulas, which use soy protein isolates as the replacement of dairy proteins, contain fairly high amount of soyasaponins ranging from 1.2 – 2.3 $\mu\text{mole/g}$ of dry formula.

Soy intake differs significantly among different ethnic populations. In Japan and China, the mean age of introduction of soy products is about 1.9 yr in the form of soy drink and tofu (Hsiao *et al.* 1999). According to Wu (1998), the estimated intake of soy products is 20 to 141 g/day in Chinese populations in Asia. The most commonly consumed soy foods in these populations were soy drinks, tofu, soybean curd jelly, miso and soy sauce. Total consumption of soy products in western countries was estimated to be 3 to 10 g/day. The most commonly consumed soy foods were tofu, soymilk, soy yogurt, tempeh, textured vegetable protein, soy nuts, vegetable-based soy burgers and soy hotdogs (Kirk *et al.* 1999). Therefore, estimated total daily intake of soyasaponins from soy consumption would be about 15 to 120 μ moles/day in Asian Chinese population and 0.7 to 6 μ moles/day in western countries. In the United States, the consumption of soy products has been increasing recently in response to the recognition of beneficial health effects of soybeans. The FDA has approved a health claim of soy protein for the reduction of the risk of coronary heart diseases. It was recommended the consumption of 25 g of soy protein per day with a heart-healthy diet to achieve the effect (Schulz 1999). This amount of soy protein would provide about 200 μ moles soyasaponins daily, provided that the soy protein was not ethanol-washed during its production. The amount of soyasaponins that people are exposed to in the diet is comparable to isoflavones. The estimated average daily isoflavone intake was 92 μ moles/day among the Japanese (Wakai *et al.* 1999) and about 0.25 to 0.87 μ moles/day for healthy western postmenopausal Caucasian women (Kleijn *et al.* 1999).

C. Human health-related properties of soyasaponins

Many studies have demonstrated health-promoting activities of soyasaponins.

Soyasaponins could help to lower blood cholesterol level (Potter *et al.* 1993; Sauvaire *et al.* 1996; Lacaille-Dubois and Wagner 1996) and inhibit growth of cancer cells (Rao *et al.* 1995; Lee *et al.* 1999). Soyasaponins might be involved in eliminating digestive toxins and strengthening the immune system (Kenarova *et al.* 1990; Uemura *et al.* 1995). Although the mechanisms for these biological activities are not fully understood, the variety of effects are believed to be associated with the amphiphilic nature of soyasaponins. Each of these potential health effects are reviewed in the following sections.

C.1 Hypocholesterolemic effect of soyasaponins

The potential of soyasaponins to lower blood cholesterol level has drawn attention recently. Clinical trials have shown that consumption of soy protein, compared to other proteins such as those from milk or meat, could lower total and LDL-cholesterol levels in the blood (Anderson *et al.* 1995). Soyasaponins, one of the primary phytochemicals in soy products, have been proposed to have the ability to lower plasma cholesterol (Oakenfull *et al.* 1990; Potter *et al.* 1995). Oakenfull (2001) proposed two mechanisms by which soyasaponins could affect cholesterol metabolism. One mechanism suggested that soyasaponins might form insoluble complexes with cholesterol in the intestine to inhibit absorption of endogenous and exogenous cholesterol. A second mechanism implied that soyasaponins might interfere with the enterohepatic circulation of bile acids by forming mixed micelles with bile salts that would block reabsorption of bile acids. Oakenfull *et al.* (1984) observed that having 1% soyasaponin extract in 1% cholesterol-containing diet increased bile acid and neutral sterol excretion in rats. Sidhu and Oakenfull (1986) reported

that an isolated crude soyasaponin fraction from soy at 1% (g/v) reduced the absorption rate of bile salts in rat intestines by forming micelles with bile acids.

However, direct observation of cholesterol-lowering after feeding soyasaponin-containing diet on animal or humans is lacking. Calvert and Blight (1981) conducted a double-blind cross-over study feeding ten male hypercholesterolemic outpatients 50 g of soy flour per day with either 22 or 4 g saponins/kg for 4 weeks. They observed no significant changes in blood lipid profile or bile acid excretion either between the two treatments or between the levels at the beginning of the study and the end of the study. Moreover, two confounding phenomena have been observed in several studies. When 1.5 to 10% soy or quillaja saponins were added to the casein-based diets, regardless of fat level in the diet, there was a significant decrease of LDL cholesterol and LDL/HDL ratio in gerbils, rats, and rabbits accompanying increased bile acids excretion. The plasma cholesterol levels were not significantly different between the treatments that were either soyasaponin-depleted soy-based diets or intact soy-based diets with extra soyasaponins added. However, the cholesterol levels were significantly lower in the animals fed the soy-based diets than those fed the casein diet (Oakenfull *et al.* 1984; Potter *et al.*; 1993; Ueda *et al.* 1996). In addition, having 1 or 10% soyasaponins in the 1% cholesterol diets was effective for lowering serum and liver cholesterol and triglyceride levels in rats and chicks in comparison with the 1% cholesterol diets without soyasaponin supplementation, but this effect was not observed if the diets contained only 0.1% cholesterol (Oakenfull *et al.* 1984; Ueda *et al.* 1996). These observations suggest that: 1) there might be a threshold of dietary cholesterol level in these animal models to achieve increased serum and liver cholesterol levels; 2) the cholesterol-lowering effect of soyasaponins is probably due to the interaction of soyasaponins with

cholesterol in the gut and consequently prevention dietary cholesterol from absorption. All these studies provide only indirect evidence that soyasaponins might be one of the factors contributing to soy's hypocholesterolemic effect. Additionally, all the soyasaponin fractions used in the above experiments were produced by alcoholic extraction, meaning the extracts also contained isoflavones and phenolic acids. Isoflavones have been shown to reduce total serum cholesterol level when added to casein diet in hamsters (Balmir *et al.* 1996), although their mechanism is not clear at this time. Interpretations of the cholesterol-lowering effect of soyasaponins are confounded by the mixed nature of alcoholic extracts of soy. Based on the results from these studies, it would be difficult at this point to conclude that soyasaponins are the primary active components in the hypocholesterolemic effect of soy products.

C.2 Anti-carcinogenic effect of soyasaponins

The epidemiological data from eastern Asian countries have suggested that consumption of soy products may be associated with the reduced risk of hormone and non-hormone dependent cancers (Messina *et al.* 1994). Among the bioactive constituents of soy, soyasaponins have been shown to significantly suppress carcinogenesis in *in vitro* and *in vivo* experiments. Konoshima *et al.* demonstrated the inhibitory effect of soyasaponins on 7,12-dimethyl-benz[*a*]anthracene (DMBA) initiated and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-promoted mouse skin tumor model in a series of experiments (1992, 1996). They observed that 85 nmol of soyasaponin I, when applied before each TPA treatment, delayed the formation of papillomas in mouse skin and significantly reduced the numbers of papillomas formed per mouse. Koratkar and Rao (1997) showed that 3% soyasaponin in the

diet reduced the incidence of preneoplastic lesions on the colon mucosal in mice initiated by azoxymethane.

Several mechanisms by which soyasaponins may act as chemopreventive agents have been postulated and investigated. Soyasaponins may have a direct cytotoxic effect on cancer cells. Sung *et al.* (1995) investigated the effect of soyasaponins on the growth and viability of HCT-15 colon carcinoma cells. Their data showed that 24 h exposure to soyasaponins at 10 to 600 ppm significantly decreased cell growth and viability in a dose-dependent manner. Several studies suggested that there was an anti-mutagenic activity for soyasaponins. Plewa *et al.* (1999) demonstrated the anti-mutagenic activity of soyasaponins on mammalian cells. They observed that a fraction (PCC100) isolated from soy molasses at 50 µg/mL repressed the genotoxicity of dietary carcinogen 2-amino-3-methyl-imidazo-(4,5-f)quinoline in human lymphocytes. This fraction also suppressed 2-acetoxyacetylaminofluorene (2-AAAF) induced DNA damage in Chinese Hamster Ovary (CHO) cells. Berhow *et al.* (2000) identified the major components in this fraction as a mixture of group B soyasaponins. Furthermore, Berhow's group found that the purified soyasapogenol B from this PCC100 fraction exhibited the most potent protective effect against 2-AAAF induced *gpt* gene mutation in CHO cells among group B soyasaponins. The mechanism of the anti-mutagenic activity of soyasaponins is not clear. Berhow hypothesized that group B soyasaponins might be antimutagenic possibly by intercepting reactive molecules inside the cells. The data from Sung and Park (1999) supported this hypothesis. Their results showed that 0.3 mg/plate of soyasaponins significantly inhibited *tert*-butylhydroperoxide-induced malonaldehyde production and increased cellular anti-oxidative enzyme activities in a human hepatocarcinoma cell model. Soyasaponins at 10-50

$\mu\text{g/mL}$ showed an inhibitory effect on the DNA-aflatoxin B1 adduct formation in cultured human colon and liver cells (Joel and Sung 1999). Soyasaponins inhibited the expression of oncogenic Epstein-Barr virus genome *in vitro* (Tokuda 1988). These results suggest that soyasaponins might be effective in reducing cellular DNA damage caused by carcinogens. Furthermore, Oh and Sung (2001) evaluated the effect of soyasaponins on cell proliferation, differentiation and apoptosis in human colon cancer cells. Soyasaponins at 150 to 600 ppm inhibited TPA-induced cell proliferation by suppressing protein kinase C in a dose-dependent manner. In this study, increased alkaline phosphatase activity was observed in the soyasaponin-treated cells, suggesting soyasaponins effectively induced differentiation of the cancer cells. Soyasaponins did not affect apoptotic activity in this study. Wu *et al.* (2001) reported that 5 to 25 μM of soyasaponin I acted as a potent, specific sialyltransferase inhibitor in a dose-dependent manner *in vitro*. Enhanced sialyltransferase activity has been associated with oncogenic transformation and tumor metastasis (Harvey *et al.* 1992; Gessner *et al.* 1993). From these observations, it is evident that soyasaponins might be effective anti-carcinogens in the initiation and promotion stages of carcinogenesis.

The inhibitory effect of soyasaponins on colon cancer development has drawn attention since saponins taken orally might not be absorbed and remain in the intestinal tract presumably. Bennink's group (2000) evaluated the potential of soy consumption to inhibit colon carcinogenesis in rats. In their study, it was found that full fat and defatted soy flour diets decreased the formation of precancerous lesions in the rats, whereas ethanol-washed soy concentrate did not reduce tumor incidence, and adding isoflavones alone to the ethanol-washed soy concentrate did not inhibit tumor incidence (Bennink 2000). These data indicated that ethanol-soluble phytochemicals other than isoflavones might have been

responsible for the cancer inhibition effect of soy observed by Bennink. Since soyasaponins are one of the primary phytochemicals found in alcoholic extract of soy, their role in colon carcinogenesis deserves further investigation due to the anti-carcinogenic potential of saponins. The inhibitory effect of 3% of soyasaponins in AIN-76 diet over 14 weeks on the formation of preneoplastic lesions on the colon mucosal of mice has been reported (Koratkarn and Rao 1997). However, it is notable that 3% soyasaponins in the diet is about ten-fold higher than the level that people would normally be exposed to through their diet since most of the soy foods contain about 0.02 to 0.5% soyasaponins (Hu *et al.* 2002). The mechanism of the colon cancer inhibiting effect of soyasaponins is not well understood. Besides the anti-carcinogenic activities of soyasaponins discussed above, it is also likely that soyasaponins may bind to primary bile acids in the gut to reduce the formation of secondary bile acids, considered colon cancer promoters (Sugezawa and Kaibara 1991).

C.3 Other health-protective activities of soyasaponins

Group B soyasaponins inhibited HIV replication and infection *in vitro*. Nakashima *et al.* (1989) demonstrated that 0.5 mg/mL soyasaponin I significantly reduced HIV-induced cytopathic effects on MT-4 cells and virus-specific antigen expression six days after infection. Soyasaponin II showed similar effects but was less potent than soyasaponin I. Soyasaponin II exerted dose-dependently virucidal activity on enveloped virus including human cytomegalovirus, influenza A virus, and HIV-1 at concentrations of 40, 200 and 1000 μM , and consequently reduced virus infectivity (Hayashi *et al.* 1997).

The anti-hepatotoxic activity of soyasaponins has been observed in a number of *in vitro* studies with a comparable amount of cell density, about 10^5 to 10^6 per culture dish.

Soyasaponin I at doses of 50 to 500 $\mu\text{g/mL}$ reduced the elevation of glutamic pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT) activities induced by CCl_4 in the primary cultured rat hepatocytes (Miyao *et al.* 1998). The structure-hepato-protective relationship of soyasaponins has been investigated by Nohara's group (1997, 1998). Alanine aminotransferase (ALT) activity was used as an indicator of immunologically induced liver injury on primary cultured rat hepatocytes. Soyasaponin I at 90 $\mu\text{g/mL}$ inhibited the increase of ALT in this experimental model and its effect appeared to be more potent than another triterpene saponin, glycyrrhizin (Arao *et al.* 1997). They also found no significant protective effect from soyasaponins I or II below 200 μM , whereas soyasaponin III and IV showed strong protection at 30 μM (Kinjo *et al.* 1998). It indicated that the composition of the sugar moiety might play an important role in hepato-protective action of soyasaponins. The disaccharide-attached soyasaponins appeared to be more effective than trisaccharide-attached soyasaponins. Ikeda *et al.* (1998) compared the hepato-protective effect of soyasapogenol B analogs from soyasaponin I using the same model. They found that at 30 μM of soyasaponin III, which has a disaccharide group, and soyasapogenol B monoglucuronide, which has a glucuronic acid group, appeared to be more effective than soyasaponin I, which has a trisaccharide group, and the aglycone, soyasapogenol B.

Recent studies have shown varied results on the anti-oxidant activity of soyasaponins. Several studies demonstrated that the DDMP moiety of soyasaponins was the actual free radical scavenger (Yoshiki and Okubo 1995; Tsujino *et al.* 1994). Yoshikoshi's group investigated whether non-DDMP soyasaponins had the ability to protect the cells

from oxidative damage (1996). In contrast, they found that 20 μM non-DDMP soyasaponins, soyasaponin I and Ab, exhibited an even greater inhibition to cytotoxicity induced by hydrogen peroxide than DDMP-conjugated soyasaponin βg in the cultured mouse fibroblasts.

Rowlands *et al.* (2002) claimed that soyasapogenols had structure similarity to estrogen and examined estrogenic activity of soyasapogenol A and soyasapogenol B by measuring their ability to stimulate proliferation of estrogen responsive human breast cancer cells. Their results revealed that soyasapogenol B was anti-proliferative and soyasapogenol A had weak estrogenic activity compared to E2 but equivalent to genistein *in vitro*.

Caution should be used in interpreting the literature since most of the potential health promoting properties of soyasaponins were observed in animal models or in *in vitro* experiments with high doses not relevant to the levels consumed by humans. The relevance of these results to humans under *in vivo* conditions is not clear. *In vivo* information is needed to elucidate and verify these properties of soyasaponins in animal and humans. Moreover, people are exposed to a complex of soy constituents instead of a single constituent of soybeans through soy-rich diet. It will be of great importance to investigate not only the independent activity of each constituent but also the interaction among these soybean constituents, such as isoflavones, soyasaponins, phenolic acid and phytosterols, on the soy-attributed health beneficial effects.

D. Toxicity of soyasaponins

Oleszek (1990) has shown saponins to be hemolytic *in vitro*. It could be a health threat if saponins enter the circulation system directly. The oral toxicity of saponins to

warm-blood animals is relatively low, probably due to their poor bioavailability (George *et al.* 1965). Few negative effects have been observed in humans experiencing long-term consumption of saponins from edible plants. Saponins from quillaja bark, licorice root, and yucca rhizome are widely used as food additives, immune adjuvants, and anti-dermatophytic ingredients in cosmetics. These crude saponin extracts are classified as 'generally recognized as safe (GRAS)' by the U.S., FDA (Osamu *et al.* 1996).

The systematic evaluation of soyasaponin toxicity is lacking. Soyasaponin I was not mutagenic up to 0.5 mg/plate in the Ames mutagenicity test (Czeczot *et al.* 1994). Saponins from gypsophylla, saponaria and soybeans are all triterpenoid saponins. Gypsophylla and saponaria saponins increased the permeability of intestinal mucosal cells of rats *in vitro* while soyasaponins appeared to be less effective (Johnson *et al.* 1986). The interaction between soyasaponins and nutrients has been indicated in several studies. Ikedo *et al.* (1996) reported that crude soyasaponins extracted from soybean hypocotyls interacted with bovine serum albumin (BSA) and increased resistance of BSA to chymotrypsin hydrolysis *in vitro*. Shimoyamada *et al.* (1998, 2000) observed that these crude soybean soyasaponins suppressed chymotryptic hydrolysis of soybean proteins and lactoglobulins *in vitro*. These findings suggested that soyasaponins might affect the digestibility and bioavailability of these proteins. Another nutritional problem associated with saponins in the diet is the interference with mineral absorption. A diet with 2% of gypsophila saponins or alfalfa saponins has been implicated in increasing fecal excretion of minerals and associated with the chronic induction of negative mineral balance in rats, while a similar level of soyasaponin I in the diet did not affect Fe and Zn absorption (Southon *et al.* 1988).

E. Metabolism and bioavailability of soyasaponins

Detailed information on the fate of saponins in the animal or human gut is generally lacking. Saponins have been considered to be poorly absorbed in animals after oral dosing and might be either excreted unchanged or metabolized in the gut. Consequently, it was difficult to demonstrate how saponins act *in vivo*, and there is no direct *in vivo* evidence for many of the biological activities associated with saponins.

Glycyrrhizin is a triterpenoid saponin from licorice with glycyrrhetic acid as the aglycone. Ishida *et al.* (1989) showed that glycyrrhizin could be partially hydrolyzed to a sugar and an aglycone portion in the rat digestive tract. Kim *et al.* (2000) showed that glycyrrhizin was metabolized by human intestinal microflora to 18 β -glycyrrhetic acid (GA), as a main product, and to 18 β -glycyrrhetic acid-3-O- β -D-glucuronide (GAMG) as a minor product. Glycyrrhetic acid, the major gut metabolite of glycyrrhizin, was absorbable and displayed anti-inflammatory and anti-hepatotoxic activities *in vivo* (Horigome *et al.* 2001; Nose *et al.* 1994). Among GA, GAMG and glycyrrhizin, GA displayed the most cytotoxic activity against tumor cell lines and the most potent inhibitory effect on rotavirus infection as well as *Helicobacter pylori* growth (Kim *et al.* 2000).

The pharmacokinetics and metabolism of ginseng saponins, a group of steroid saponins, have been evaluated. Takino's group (Odani *et al.* 1983) reported that 2% of ingested ginsenosides with a disaccharide group were absorbed from the intestinal tract of rats, whereas only 0.1% of ingested ginsenosides with a trisaccharide group were absorbed. Therefore, these steroid saponins might have very low bioavailability *in vivo*. However, these saponins were reported to be metabolized by intestinal microbes possibly due to β -glucosidase activity. Takino's group found that the oral dose of ginsenoside Rb2 was barely

metabolized in the gastric juice of rats and only underwent slight oxygenation (Karikura *et al.* 1991). However, six metabolites of ginsenoside Rb2 were identified in the large intestine content after oral dosing in rats. These metabolites were also found *in vitro* when Rb2 was incubated with rat cecal contents (Karikura *et al.* 1990). The pattern of ginsenoside degradation in the rat intestine showed that decomposition began with the cleavage of the terminal glucose residue of the oligosaccharide attached to C-3 or C-20 hydroxyl group of the aglycone. Then the hydrolysis proceeded stepwise to liberate the secondary products, metabolite I, II, III, IV, V and XII (Figure 4). Ginseng saponin metabolism in humans was investigated by Hasegawa *et al.* (1996). Ginseng saponins showed a similar degradation pattern by human intestinal bacteria *in vitro* when compared to the pattern observed in rats. *Prevotella oris*, one of the bacterial species capable of metabolizing ginsenosides, was isolated in human fecal specimens (Hasegawa *et al.* 1997). However, no ginsenosides or their microbial metabolites were detected in the urine or blood after a dose of 150 mg ginseng saponins/kg body weight was ingested by a human subject. It is noteworthy that the presence of ginsenosides and/or their microbial metabolites in the feces of this subject were not examined. Thus, there is no direct evidence whether ingested ginseng saponins could be metabolized in the intestinal tract *in vivo*.

The metabolism of soyasaponins in humans has not been well characterized. Gestetner *et al.* (1968) incubated the content of cecum and colon of rats, chicks and mice with soyasaponins *in vitro* and detected both soyasaponins and soyasapogenols in the culture after 3 h of anaerobic incubation. The saponin-hydrolyzing enzymes purified from the cecal microflora of rats were identified as nonspecific glycosidases, which were able to liberate glucose, galactose, arabinose, rhamnose and glucuronic acid from soyasaponins after *in vitro*

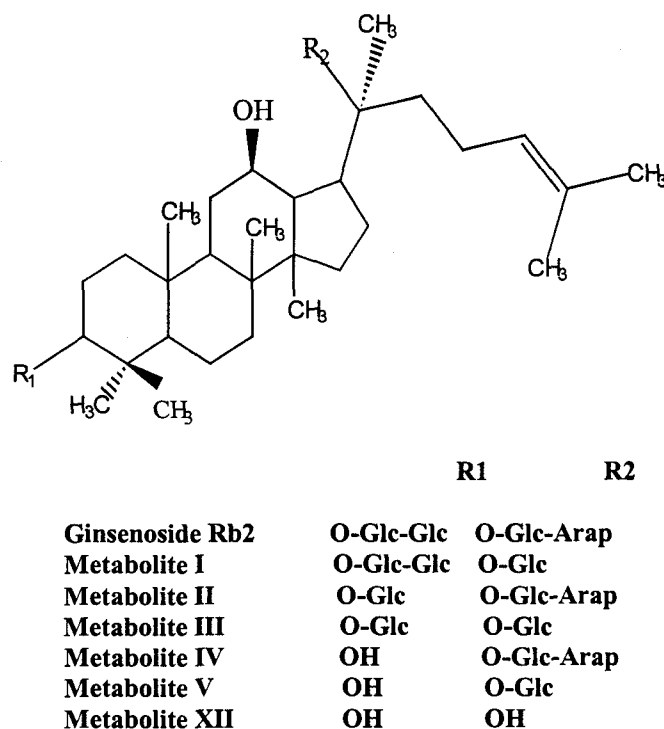


Figure 4. Structures of ginsenoside Rb2 and its intestinal metabolites (Karikura *et al.* 1990). Glc: glucopyranosyl; Arap: arabinopyranosyl

incubation. They suggested that mammalian gut microbial enzymes had the ability to hydrolyze various glycosidic bonds of soyasaponins to liberate aglycones. Human intestinal bacteria, especially *Lactobacilli*, *Bacteroides* and *Bifidobacteria* species, possess glycosidase and β -glucuronidase activities (Rowland *et al.* 1970; Hawksworth *et al.* 1971). These bacterial species may play a role in hydrolyzing sugar-conjugated soyasaponins in the human intestinal tract. The average length of time during which food residue stays in the large intestine is about 24 h or longer. The microbes in the large bowel would have sufficient time to interact with soyasaponins and perform their hydrolysis activities. Furthermore,

soyasaponins, either in the intact or partially hydrolyzed forms, might remain in the intestine long enough to exert their actions. Soyasaponin absorption was examined as well after oral dosing of these animals in Gestetner's study. Since there was no reliable method to quantify soyasaponins at that time, soyasapogenols and soyasaponins were determined qualitatively in the digestive tracts and the blood samples of these animals using TLC and hemolysis analysis. Neither soyasaponins nor soyasapogenols were found in the urine or blood samples, suggesting that soyasaponins might not be absorbed in these animals. However, there is no direct evidence to demonstrate absorbability and pharmacokinetics of soyasaponins in animals or humans.

F. Influence of gut microflora on metabolism and bioavailability

It is well established that the microbial community that inhabits the human large intestine plays an important role in metabolizing a variety of xenobiotics, thus potentially affecting the bioavailability and/or altering the activity and toxicity of ingested phytochemicals (Boxenbaum et al. 1979; Rowland 1998). The gut microflora possess a diverse range of metabolic activities to catalyze reactions including reductions, hydrolyses, hydroxylations, degradations and syntheses (Rowland 1988). Many plant glycosides ingested in the normal human diet are hydrophilic with relatively high molecular weight, such as flavonoids and cyanogenic glycosides. These compounds were considered to be poorly absorbed by the small intestine and pass largely unaltered into the lower bowel where they could be subjected to metabolism by the intestinal microflora (Rowland 1970). Gastrointestinal microbial metabolism often results in the formation of more lipophilic metabolites of ingested glycosides due to hydrolysis or reduction of glycosidic bonds,

deconjugations, dehydroxylations and decarboxylations (Boxenbaum *et al.* 1979).

Microbial metabolism may be responsible for the prolonged retention of ingested substances or metabolites in systemic circulation due to possible regeneration of parent compounds from conjugated metabolites in the intestine through enterohepatic recirculation.

There are a number of studies investigating the effect of the gut microbes on the bioavailability and metabolism of phytochemicals. Hollman *et al.* (2001) found that quercetin, a flavonoid present in plants, was absorbed twenty times more in quantity and ten times faster if ingested as quercetin-glucose than as quercetin-rutinoside. The authors suggested that quercetin glucoside might be actively absorbed from the small intestine, whereas its rutinoside might be absorbed from colon after deglycosylation by gut microflora. Kim *et al.* (1998) revealed that rutin, hesperidin, naringin and poncirin were converted into their aglycones by the bacteria producing α -rhamnosidase and β -glucosidase or endo- β -glucosidase, while baicalin, puerarin and daidzin were converted into their aglycones by the bacteria producing β -glucuronidase, C-glycosidase and β -glycosidase, respectively. Grolier *et al.* (1998) demonstrated that the bioavailability of α - and β -carotenes could be significantly improved in rats by reduction of gut microflora. Phytosterols in the diet are considered to be poorly absorbed in the small intestine, and consequently concentrated in the large intestine. The carbonaceous 17-side chain of phytosterols could be cleaved off by intestinal microbial (Roy *et al.* 1991). Oxidation – reduction at C3- hydroxyl group and hydrogenation of the Δ^5 double bond of phytosterols by human fecal bacteria is common (Song *et al.* 2000). These gut metabolites of phytosterols were then excreted in the feces (Weststrate *et al.* 1999).

Gut microbial metabolism of isoflavones play an important role in determining the magnitude of isoflavone bioavailability. Liu and Hu (2002) evaluated the intestinal absorption and bioavailability of genistein using a Caco-2 cell culture model and a perfused rat intestinal model. They reported that genistein was well absorbed in both intestinal models, and suggested that the observed low bioavailability of genistein *in vivo* was probably not due to poor absorbability but extensive metabolism in the intestine. Setchell *et al.* (2002) showed that soy isoflavone glucosides were not absorbed intact across the intestine enterocytes, suggesting that their bioavailability might require initial hydrolysis by intestinal β -glucosidase before entering the circulation. Isoflavone aglucones might be further degraded by gut microflora. Daidzin was metabolized to daidzein, and then to equol and O-desmethylanangolensin (ODMA) by human gut microflora (Kim *et al.* 1998; Chang and Nair 1995). Gut microbial metabolism of genistein and glycitein has not been well characterized to date. Xu *et al.* (1995) showed the overall bioavailability of daidzein and genistein varied among individuals and apparently was inversely correlated with the extent of gut microbial degradation of isoflavones. Their results were extended by Zheng (2000). Zheng reported that three-fold greater bioavailability of genistein was observed in the Chinese subjects with a relatively low degradation of genistein by fecal microflora compared to the high isoflavone degraders.

The differences in gut microflora population and bacterial enzyme activities would lead to different microbial metabolism of phytochemicals. There are many factors that influence gut microflora populations and enzyme activities, such as genetic factors, dietary factors, physical activities and gut peristalsis (Rowland *et al.* 1988). The genetic background of individuals might have some influence on gut microflora population and

metabolism. Mitsuoka *et al.* (1982) observed a higher ratio of anaerobes to aerobes and fewer enterococci in the feces of humans in the U. S. in comparison with the humans in India. Zheng (2000) observed that Chinese and Caucasians subjects significantly differed in their population distribution for daidzein degradation by gut microbes. However, it would be difficult to attribute this difference solely to genetic background of individuals, because dietary habits, cultural and environmental factors may play roles as well. The effect of diet on gut microflora and their metabolic activities has been reviewed by Rowland and Mallett (1970). High cholesterol and high meat diets are reported to significantly increase intestinal total anaerobic microflora including *Bacteroides*, *Bifidobacteria*, *Peptococci* and *Lactobacilli* species, and induce microbial β -glucuronidase activity (Reddy *et al.* 1973 and 1974). Finegold *et al.* (1974; 1983) reported that the adult Japanese diet resulted in an adult microbiota that differed from that of adults consuming a western-style diet. In an isoflavone bioavailability study, Zheng (2000) observed that Chinese subjects apparently consumed more cholesterol and red meat in their diet in comparison with Caucasian subjects. Zheng *et al.* suggested that the high meat and high fat diets might increase β -glucuronidase activity of the gut microflora. Thus, the conjugated isoflavone aglycones excreted in the bile could be hydrolyzed by gut microflora to liberate more aglycones for reabsorption, which might contribute to the high bioavailability of isoflavones in the Chinese subjects. The profile of intestinal microbiota remain relatively unchanged once it is established changes provided that there is no significant change in dietary habit and health status (Stark and Lee 1982). However, there is shift in the composition of the intestinal microflora when aging. In elderly persons, bifidobacteria decrease or disappear, while lactobacilli, enterococci, enterobacteria and clostridia increase (Holm 2003).

G. Using Caco-2 cell model to assess intestinal absorption and bioavailability of phytochemicals

People are exposed to a variety of phytochemicals through diet. These phytochemical molecules may have to be absorbed from the gastrointestinal tract and enter the systemic circulation in sufficient quantities to exert their activities. Therefore, it is crucial to understand the absorption and bioavailability of these phytochemicals. Currently, there are a number of *in silico*, *in vitro*, *ex vivo* and *in vivo* approaches to predict permeability, absorbability and gastrointestinal metabolism of these molecules. Pelkonen *et al.* (2001) compared the common absorption and bioavailability models currently in use (Table 3).

Cell culture has been used more widely in nutritional and pharmaceutical research to compensate human and animal models due to financial and ethical considerations. Several cellular models are available to study the absorption characteristics of xenobiotics. Two of the most widely used models are Caco-2 cells in monolayer and MDCK (Maudin-Darby canine kidney) cells in monolayer. Caco-2, a cell line from human colon adenocarcinoma, was established by Jorgen Fogh in 1974 from a 72-yr old Caucasian man (Fogh *et al.* 1977). MDCK cells were derived from a kidney of an adult female cocker spaniel and established by Madin and Barby in 1958 (Gaush *et al.* 1966).

A potential advantage of Caco-2 cells is that they are human enterocyte-derived with a microvillous surface. As reviewed by Meunier *et al.* (1995), Caco-2 cells spontaneously differentiate into a highly polarized continuous monolayer after reaching confluence with functional tight cellular junctions. Well-developed brush border microvilli and an undisturbed water layer found on the apical surface resemble the properties of mature

Table 3. Common absorption and bioavailability models currently in use (Pelkonen *et al.* 2001)

System	Model	Advantages	Disadvantages
<i>In vitro</i>	Artificial membrane systems such as immobilized artificial membrane	High throughput, easy to use, analytically easy	Measure only transcellular permeation, lack of active transport
	Cell culture models such as Caco-2, MDCK	Moderate to high throughput of drug screening, Measure active and passive transport Human epithelium for Caco-2 cells	Labor intensive, analytically more difficult, intra – and inter-lab variability present
	Brush border membrane vesicles	Moderate throughput, Measure active and passive transport	Labor intensive, analytically more difficult, intra – and inter-lab variability present
<i>In situ</i>	Rat intestinal perfusion	Close to <i>in vivo</i> situation, Have transporters, enzymes and relevant tight junctions	Labor intensive, species differences, possible effects of manipulations, technically challenging
<i>In vivo</i>	Rat portal vein models	Close to <i>in vivo</i> situation, Have transporters, enzymes and relevant tight junctions Have presystemic metabolism	Labor intensive, species differences, possible effects of manipulations

intestinal absorptive mucosal cells with a high level of associated enzyme activities such as disaccharidases, peptidase, and alkaline phosphatase (Arthursson 1989; Hidalgo *et al.* 1989). These aspects of Caco-2 cells can be used to assess the rate and extent of absorption of xenobiotics. It has been found that Caco-2 cells express *P*-glycoprotein, which is expressed along the entire gut and works as an efflux pump that extrudes a wide range of structurally diverse substances from the cell. Thus, Caco-2 cells can be used to measure bi-directional transport of the substances. Differentiated Caco-2 cells have the ability to express cytochrome P450 CYP3A4 and phase II enzymes such as UDP-glucuronosyltransferase, sulfotransferase and glutathione-*S*-transferase so that this model could be used to study presystemic metabolism by intestinal enterocytes as well (Caro *et al.* 1995). Caco-2 cell models have also been used to study cholesterol absorption and the hypocholesterolemic effect of phytosterols because the cells displayed the ability to uptake micellar sterols and esterify sterols inside cells (Schulthess *et al.* 1996; Compassi *et al.* 1997).

Using Caco-2 cell models, many studies have been performed to demonstrate the uptake, transport and accumulation of nutrients and pharmaceutical agents using microporous membrane inserts. However, the disadvantages of this system include that they need long culturing times (2-3 weeks) to express fully differentiated functions, the cells form very tight junctions in monolayer, and exhibit a high transepithelial resistance relative to that *in vivo* (Pelkonen *et al.* 2001). Additionally, Barthe *et al.* (1999) suggested that the Caco-2 cell system was static, and gave low rates of transport, and exaggerated the paracellular route compared to the small intestine. Lennernas (1997) summarized correlations of permeability from different *in vitro* transfer models with human data. He found that the permeability of the compounds via passive transport in Caco-2 monolayers

were comparable to those seen in the human colon, whereas the permeability of large hydrophobic compounds and carrier-mediated transported compounds were much lower in Caco-2 cells than in the human jejunum. The lower permeability in Caco-2 models might be partially due to a lower paracellular and/or a larger intervillous area available *in vivo* (Schwartz *et al.* 1995). Furthermore, we have to bear in mind that Caco-2 cells are cancerous cells in spite of their similarity to normal intestinal cells to some extent. It is possible that these cells may behave differently from the normal cells somehow. These factors should be taken into account when we interpret the absorbability data from Caco-2 cell model and extrapolate to humans.

The Caco-2 cell model has been widely used to measure absorbability of a variety of phytochemicals. Flavonoids can be used as an example. It is known that flavones and isoflavones can be found as conjugated aglucone forms in the systemic circulation after oral administration of their glycosides, either as plant products or as pure compounds. However, several studies have shown that glucosides, such as daidzin, genistin and quercetin, were not absorbed across Caco-2 cell monolayer (Walle *et al.* 1999; Walgren *et al.* 1998; Steensma *et al.* 1999). Kuo (1998) demonstrated that ^{14}C -flavone was transported across Caco-2 monolayer rapidly in both luminal-to-basolateral and basolateral-to-luminal directions, indicating the aglucones of flavonoids were absorbable by isolated intestinal epithelial cells. Oitate *et al.* (1998) showed that apical-to-basolateral transport of genistein across Caco-2 monolayer was significantly greater than in the opposite direction. The transport could be inhibited by the presence of other flavonoids such as rutin and quercetin. The authors suggested that transport of genistein might be a carrier-mediated process, which might transport rutin and quercetin as well. Flavonoids could be not only transported but also

metabolized by intestinal epithelial cells. Using a Caco-2 cell culture and a perfused rat intestinal model, Liu and Hu (2002) found that the vectorial transport of genistin and apigenin favored excretion by Caco-2 cells. In addition, 1 to 2% of total genistin and apigenin applied in the apical chamber were metabolized to their aglucones by Caco-2 cells. In contrast, genistein and apigenin showed four times greater transport than their glucosides, and their absorptive transport was as same as the secretory transport in Caco-2 cell model. The glucosidic hydrolysis and glucuronide conjugation of flavonoids were also observed in a perfused rat intestine model system. Steensma *et al.* (1999) reported sulfate and glucuronide conjugation of genistein in Caco-2 cell model and rat perfused gut segments. Therefore, the authors suggested that intestinal disposition of these flavonoids might be a complex of absorption, metabolism and efflux processes besides gut microflora metabolism.

Bioavailability and absorption data for saponins is scarce. Although Caco-2 cell model has been widely used to evaluate absorbability and pre-systemic metabolism of many nutrients and phytochemicals, no study has reportedly used this model to measure saponin absorbability. Chao *et al.* (1998) observed that 0.01 – 0.1% quillaja saponin DS-1 in the culture medium reduced transepithelial electric resistance of Caco-2 cell monolayer and increased permeability of mannitol and d-decapeptide without causing any detectable morphological changes of the monolayer. The integrity and viability of Caco-2 cell monolayer was recoverable after removing DS-1. This data suggested that saponins might have the ability to increase the permeability of Caco-2 cell monolayer while exhibiting only a low adverse effect on the epithelial viability and barrier function. Caution should be taken when we evaluate absorbability of saponins using Caco-2 cell model. The confluence of the cell monolayer should be carefully monitored before and after saponin treatment.

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METABOLISM OF SOYASAPONIN I BY HUMAN INTESTINAL MICROFLORA

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ABSTRACT

The metabolism of soyasaponin I (3-*O*-[α -*L*-rhamnopyranosyl- β -*D*-galactopyranosyl- β -*D*-glucuronopyranosyl]-olean-12-en-3 β , 22 β , 24-triol) by human gut microorganisms was investigated in order to elucidate the metabolism of dietary soyasaponins in the human intestine. Fifteen healthy women aged 18 to 52 years consumed a soy-free diet for 5 days. Fresh stools were collected and incubated in a brain-heart-infusion media with 10 μ mol soyasaponin I/g feces under anaerobic conditions at 37°C for 48 h. The disappearance of soyasaponin I in this *in vitro* fermentation system displayed an apparent first-order rate loss kinetics over 48 h. Two distinct soyasaponin I degradation phenotypes were observed among the subjects: rapid soyasaponin degraders with rate constant $k = 0.24 \pm 0.04 \text{ h}^{-1}$, and slow degraders with $k = 0.07 \pm 0.02 \text{ h}^{-1}$. There were no significant differences in the subjects' body mass index, stool moisture, gut transit time and soy consumption frequency between the two degradation phenotypes. The subject distribution between two soyasaponin metabolic phenotypes was not significantly different between the two ethnic groups: the Asian subjects and the Caucasian subjects ($P = 0.07$). Two primary gut microbial metabolites of soyasaponin I produced in this fermentation system were soyasaponin III (3-*O*-[β -*D*-galactopyranosyl- β -*D*-glucuronopyranosyl]-olean-12-en-3 β , 22 β , 24-triol) and

soyasapogenol B (*4-methoxyl-olean-12-en-3 β , 22 β , 24-triol*). Soyasaponin III was generated during the first 24 h incubation and disappeared by 48 h. Soyasapogenol B appeared to be the final metabolic product during the 48 h anaerobic incubation. These results suggest that dietary soyasaponins can be metabolized by human intestinal microflora. The sugar moieties of soyasaponins appeared to be hydrolyzed sequentially to yield the smaller and more hydrophobic metabolites.

Keywords: soyasaponins, soyasaponin metabolism, intestinal microflora, phytochemicals

INTRODUCTION

Soyasaponins are a group of the secondary metabolites found in variety of leguminous plants (Okubo *et al.* 1996). The basic structure of soyasaponins is a triterpenoid aglycone attached to one or two polysaccharide chains. Significant amounts of soyasaponins are found in soybeans and soy products at concentrations between 0.5 to 114 $\mu\text{mol/g}$ (Hu *et al.* 2002). The potential health-promoting properties of soyasaponins have been demonstrated in many studies. Soyasaponins have been considered as the main contributors to the cholesterol-lowering effect of soy consumption (Oakenfull *et al.* 1990; Potter *et al.* 1995). This effect might be achieved by soyasaponins binding to bile acids and cholesterol in the intestine to reduce their reabsorption (Oakenfull 2001). Soyasaponins showed the anti-carcinogenic activity against various tumors or tumor cell lines (Rao *et al.* 1995; Konoshima *et al.* 1996; Koraktar *et al.* 1997; Oh and Sung 2001). Soyasaponins have also been shown to possess hepato-protective and anti-viral activities (Miyao *et al.* 1998; Kinjo *et al.* 1998; Hayashi *et al.* 1997).

It is important to understand the pre-systemic metabolism and absorption of soyasaponins because soyasaponins are generally taken by humans through soy foods. The forms of soyasaponins that are most likely to be involved in the potential health effects of these compounds are not known. Most of the studies investigating the biological effects of soyasaponin were limited to *in vitro* experiments and a few animal studies. Data on the bioavailability and absorption of saponins is scarce. Gestetner *et al.* (1968) reported that ingested soyasaponins were hydrolyzed to aglycones by non-specific glycosidases of cecal microflora in chicks, rats and mice. Karikura *et al.* (1990) observed that ginseng saponins were decomposed into the smaller molecules in the rats intestine via hydrolysis of the sugar moieties. However, no study has reported the fate of soyasaponins nor explored their possible metabolites in human intestine to date.

The present study was designed to investigate whether soyasaponins were metabolized by human intestinal microflora, to identify the possible gut metabolites of soyasaponins. Soyasaponin I (Scheme 1), as a representative of soyasaponins, was tested in the study because it is a dominant form of soyasaponins in the heat-treated soy products. Individual variability in soyasaponin metabolism by gut microflora was also evaluated and soyasaponin catabolic phenotype among the human subjects was characterized. Gut microorganism populations and bacterial enzyme activity have been known to be affected by factors such as genetic background, dietary habits and physical activity (Rowland *et al.* 1970). Therefore, the influence of individual status, such as body composition, ethnicity, gut transit time and soy consumption on gut microbial metabolism of soyasaponins, was examined as well. The information obtained in this study will help to predict soyasaponin

bioavailability in humans and provide evidence of the presence of soyasaponin metabolites in the human intestine.

MATERIALS & METHODS

Preparation of soyasaponin I, III and soyasapogenol B standards

Soyasaponin I was isolated from soy germ donated by Schouton USA, Inc. using the method of Hu *et al.* (2002). Soyasaponin III was produced by hydrolyzing 100 mg of soyasaponin I in 1 *N* hydrochloric acid-dioxane (1:1, v/v) by refluxing for 1 h. The reaction mixture was neutralized by 10 *N* sodium hydroxide and desalted with a high capacity C₁₈ SPE column (Alltech Associates Inc., Deerfield, IL) sequentially eluting with water and methanol. Crude soyasaponin III was obtained in the methanol fraction. Forty-three mg of soyasaponin III were obtained by further purification with a semi-preparative HPLC system as previously described by Hu *et al.* (2002). Soyasapogenol B was produced by hydrolyzing 100 mg of crude group B soyasaponins generously provided by Dr. Mark Berhow (National Center for Agricultural Utilization Research, USDA) in 3 *N* hydrochloric acid by refluxing for 3 h. The reaction mixture was neutralized, desalted as described above and then purified with the semi-preparative HPLC system using 70 % aqueous acetonitrile with 1 mM ammonia acetate at flow rate 2 mL/min. Total 24 mg of soyasapogenol B was yield as white amorphous powder.

The structural identity of the purified soyasaponin standards was confirmed by electrospray ionized (ESI) mass spectroscopy and ¹H-, ¹³C- NMR analyses. ESI spectra were acquired in the positive Q1MS mode on a Finnigan TSQ 700 triple quadrupole mass spectrometer (Finnigan MAT, San Jose, CA) fitted with a Finnigan ESI interface. The ESI

spectra of individual compounds gave a primary ion peak at m/z 944 $[M+H]^+$ for soyasaponin I, m/z 891 $[M+Na]^+$ for soyasaponin III, and m/z 459 $[M+H]^+$ for soyasapogenol B. 1H NMR and ^{13}C Attached Proton Test (APT) NMR spectra were acquired on a Varian VXR-300 spectrometer (Varian Inc., Palo Alto, CA). The samples were dissolved in chloroform- d_6 or dimethyl sulfoxide- d_6 (Cambridge Isotope Laboratories, Inc., Andover, MA) with tetramethylsilane (Cambridge Isotope Laboratories, Inc., Andover, MA) as an internal standard. The spectra obtained for these purified compounds were in good agreement with those reported by Baxter (1990) and Kudou *et al.* (1993).

Anaerobic incubation of soyasaponin I with human fecal microflora

The subjects were 15 healthy women recruited from Iowa State University and the surrounding community. They were aged 18 to 52 years, with body mass index (BMI) of $23.0 \pm 5.8 \text{ kg/m}^2$, and without using any medication for 3 months prior to and during the study. The subjects included ten Caucasians, four Chinese and one Indian. The study protocol was reviewed and approved by Iowa State University Human Subjects in Research Committee in 2001.

Brain-heart infusion media (BHI) used in the study was prepared with the following composition according to Zheng (2000): 100 mL BHI media contained 3.7 g of brain-heart infusion (DIFCO Laboratories, Detroit, MI), 0.4% sodium bicarbonate, 0.025% cysteine sulfide and 1 ppm resazurin (Sigma, St. Louis, MO). Soyasaponin I was added to BHI media to give a concentration of $1.6 \text{ } \mu\text{moles/mL}$. All media was saturated with CO_2 and autoclaved.

The subjects were instructed to avoid soyasaponin-containing foods listed in Appendix 1 for 4 d as wash-out period. One fresh stool from each subject was collected into sterile zip-lock bags at the end of the wash-out period. The stools were manually homogenized in the sealed bag. Ten grams of the feces was added to 25 mL sterilized BHI media and mixed by vortexing. A 10.0 mL of fecal suspension was inoculated into 25.0 mL BHI media containing soyasaponin I to give an initial concentration of 10 μ moles soyasaponin I /g feces. The mixture was incubated under anaerobic condition at 37°C for 48 h. Parallel incubation was run without soyasaponin I present in the media for each subject as a negative control. Two types of positive controls were run as follow: BHI media containing the same initial concentration of soyasaponin I was cultured without fecal material; and BHI media containing soyasaponin I was cultured with the autoclaved fecal suspension. Aliquots were taken at 0, 4, 8, 12, 24, 36 and 48 h intervals from the cultures and immediately frozen at – 20 °C.

The samples were thawed to room temperature (RT) before soyasaponin analysis. A 2.0 mL aliquot of each sample was dispersed in 8 mL methanol in a 15-mL polypropylene centrifuge tube and shaken at RT for 30 min. The suspension was centrifuged at 3000 rpm for 10 min. The supernatant was removed and the precipitate was resuspended in 10 mL methanol. After vortexing for 15 min, the sample suspension was centrifuged again. After the second centrifugation, the two supernatants were combined and evaporated to dryness under reduced pressure at RT. The residue was resuspended in 5 mL of 20% methanol and loaded onto a pre-conditioned Sep-Pak cartridge (classic short-body C₁₈, Waters Corp. Milford, MA). The cartridge was washed with 5 mL of 5% methanol. Soyasaponin was then eluted with 2.0 mL HPLC grade methanol. The extract was vortexed and subject to thin

layer chromatography (TLC) and high performance liquid chromatography (HPLC) analysis. All the samples were extracted and analyzed in duplicate. The logarithm of remaining soyasaponin I concentration was plotted versus incubation time. The reaction rate loss constant k and half-life $t_{1/2}$, were calculated according to Atkins and Jones (1997).

TLC analyses were performed on the silica gel LK6F plates (Whatman, Hillsboro, OR). Each set of the incubation samples per subject was analyzed using two different solvent conditions: one was developed with butanol-ethanol-ammonia (5:5:4, v/v); the other was developed with hexane-ethyl acetate (2:1, v/v). Soyasaponins were detected by spraying acetic acid-sulfuric acid-anisaldehyde (100:2:1, v/v) and heating at 120 °C for 10 min.

Soyasaponin I concentration and metabolite formation was determined by HPLC using the method previously reported (Hu *et al.* 2002). The mobile phases were 0.05% trifluoroacetic acid in water (solvent A) and acetonitrile (solvent B). The gradient elution was carried out as: solvent B held at 37% for 3 min, then increased from 37 to 40% in 12 min, then solvent B increased to 48% in 25 min, and finally solvent B increased to 100% in 1 min and remained at 100% for 2 min. The gradient program recycled back to the initial state of 37% solvent B in 5 min. The column temperature was 30 °C. The injection volume was 50 μ L. The flow rate was 1 mL/min and the UV absorbance was monitored from 190 to 350 nm. This HPLC program was designated as program 1.

In order to monitor the formation of more hydrophobic metabolites in the culture, the samples were analyzed with the same HPLC system using a different gradient program, designated as program 2: solvent B increased from 73 to 100% linearly in 35 min, then solvent B recycled back to 73% in 4 min.

Identification of soyasaponin I metabolites

The metabolites of soyasaponin I formed in the culture were isolated as described below. Five grams of fresh collected human feces were anaerobically incubated in the 50 mL BHI media containing 50 mg of soyasaponin I at 37 °C for 48 h. Two 25 mL aliquots were taken at 12 and 48 h. Each sample was fractionated with a high capacity C₁₈ SPE column by successively eluting with water, 30% aqueous methanol and 100% methanol. The eluted fractions containing metabolites were further separated on the preparative TLC (PK6F, Whatman, Hillsboro, OR) to give metabolites I (12 mg) and II (20 mg). The metabolites were analyzed by analytical HPLC and TLC. Their R_f values on TLC and retention times on HPLC were compared with the authentic standards.

The fecal incubation samples at each time point from the selected subjects were analyzed by LC - Atmospheric Pressure Chemical Ionization (ApCI) – MS to further confirm the chemical identity of the metabolites. The analyses were conducted on a Hewlett Packard HPLC system (Agilent Technologies, Wilmington, DE) coupled with a triple quadrupole LC-MS-MS mass spectrometer (VG Biotech). Sample separation was carried out under the same HPLC gradient programs as described above except the flow rate was 0.525 mL/min. The injection volume ranged from 5 to 20 µL depending on the concentration of the analytes in the solution. The effluent was delivered to the electrospray source configured with a corona discharge pin. Nitrogen gas was used as nebulizing and auxiliary gas for the mass spectrometer. The parameters applied to MS were: corona discharge voltage 3 kV; cone voltage 30 V; ApCI probe temperature 350 °C; source temperature 120 °C; scan time 2 sec and interscan time 0.1 sec. The full scan mass spectra over m/z range of 200 to 1100 amu were acquired on the eluted analytes. Before analysis, the mass spectrometer was tuned and

calibrated for the range of m/z 200 to 1100. The soyasaponin standards were dissolved in methanol and injected to obtain the authentic mass spectra prior to sample analysis.

Statistical analysis

All results were reported as mean \pm SD. Statistical analyses were performed with SAS system (Version 8.1, SAS Institute Inc., Cary, NC). The kinetics of soyasaponin I metabolism was analyzed by general linear regression. The phenotypic pattern in human subjects was identified by average linkage cluster analysis (Johnson and Wichern 2002). General linear model was used to analyze the differences in BMI, stool moisture, gut transit time and soy consumption frequency between two soyasaponin degradation phenotypes. The Chi-square test was performed to determine the difference in ethnicity distribution between two soyasaponin degradation phenotypes. Statistical significance was set at $\alpha = 0.05$ for all the analyses.

RESULTS AND DISCUSSION

The LC-MS chromatograms of standard soyasaponin I (3-O-[α -L-rhamnopyranosyl- β -D-galactopyranosyl- β -D-glucuronopyranosyl]-olean-12-en-3 β , 22 β , 24-triol), soyasaponin III (3-O-[β -D-galactopyranosyl- β -D-glucuronopyranosyl]-olean-12-en-3 β , 22 β , 24-triol) and soyasapogenol B (4-methoxyl-olean-12-en-3 β , 22 β , 24-triol) are shown in Figure 1 (A, B and C). Their ESI mass spectra and NMR data described in Material and Methods section were in good agreement with those reported by Baxter (1990) and Kudou et al. (1993). The mass spectra of these standards by LC-APCI-Mass spectrometer are shown in Figure 2.

Their retention times, Rf values and fragmentation pattern on ApCI mass spectrometer are summarized in Table 1.

The metabolism of soyasaponin I by intestinal microflora was examined using an *in vitro* static fecal fermentation model. The TLC profile for soyasaponin I incubation over 48 h showed that soyasaponin I gradually disappeared and two major metabolites with higher Rf values appeared in the culture over 48 h (Figure 3). This indicates that soyasaponin I was converted to more hydrophobic metabolites I and II. Metabolite II appeared to be the primary product in the culture after 24 h incubation. Disappearance of soyasaponin I was not observed in the culture inoculated with autoclaved fecal material or without fecal material (Figure 4), indicating that degradation of soyasaponin I was caused by the viable fecal microorganisms and was probably enzymatic in nature.

The HPLC chromatograms of fecal culture at 12 and 48 h are shown in Figure 1D and 1E, respectively. Metabolite I was detected along with soyasaponin I using the gradient program 1. The retention time of soyasaponin I was 25.9 min on HPLC, Rf value of 0.60 on TLC when using butanol-ethanol-ammonia solvent system. Metabolite I showed a retention time of 29.8 min on HPLC and Rf value of 0.63 on TLC. The scanned mass spectrum showed peaks at m/z 797.3 $[M+H]^+$, 599.4 $[M-rhm-gal-2H_2O+H]^+$, 441.3 $[aglycone-OH]^+$, 423.3 $[aglycone-OH-H_2O]^+$, and 405.3 $[aglycone-OH-H_2O]^+$. Metabolite II was detected on HPLC using gradient program 2. It showed a retention time of 27.4 min on HPLC, and Rf value of 0.43 on TLC when using the hexane-ethyl acetate solvent system. The scanned ApCI mass spectrum of metabolite II showed the peaks at m/z 441.3 $[aglycone-OH]^+$, 423.3 $[aglycone-OH-H_2O]^+$, and 405 $[aglycone-OH-H_2O]^+$. The HPLC and TLC chromatographic

data with the MS spectra of metabolites I and II matched those of soyasaponin III and soyasapogenol B, respectively.

These results suggest that the main metabolic pathway of soyasaponin I is the hydrolysis of terminal rhaminose off the sugar chain of soyasaponin I to produce soyasaponin III, then further hydrolysis of remaining sugars to yield soyasapogenol B. However, soyasapogenol B-monoglucuronide, one of the hypothesized metabolites of soyasaponin I, was not observed during incubation in our study. It could be explained by the higher gut microbial glucuronidase activity than rhaminosidase activity (Jang and Kim 1996). The hydrolysis of glucuronic acid off the aglycones might occur so quickly that soyasapogenol B-monoglucuronide might not be present long enough to be detected. Based on these data, it is reasonable to infer that other individual soyasaponins could be metabolized by gut microbes similarly, i.e. the terminal sugars could be cleaved off stepwise and the aglycones with structural characteristics of each soyasaponin would be produced eventually.

The metabolism of saponins by microbes has been reported. Makkar *et al.* (1997) demonstrated that quillaja triterpenoid saponins were degraded when incubated with cattle rumen liquor *in vitro* and implied their apparent metabolism by rumen microbes. Gestetner *et al.* (1968) incubated the content of cecum and colon of rats, chicks and mice with soyasaponins *in vitro* and detected both soyasaponins and soyasapogenols in the culture after 3 h incubation. However, they did not report whether any intermediate decomposition products of soyasaponins were detected. Hasegawa *et al.* (1996 and 1997) reported the metabolism of ginseng saponins by human fecal flora under anaerobic condition. They demonstrated that ginsenosides were converted into smaller molecules in the reaction

proceeding stepwise via cleavage of terminal sugars. Our results demonstrate that human intestinal microflora have the ability to metabolize soyasaponins as well. Gestetner *et al.* (1968) showed that the non-specific glycosidases purified from rat cecal microflora were able to liberate glucose, galactose, arabinose, rhamnose and glucuronic acid from soyasaponins during incubation. These data indicate that mammalian gut microbial enzymes have the ability to hydrolyze various soyasaponin glycosidic bonds. Human intestinal bacteria especially *Lactobacilli*, *Bacteroides* and *Bifidobacteria* species, generally possess glycosidase and β -glucuronidase activities (Rowland *et al.* 1970; Hawksworth *et al.* 1971). α -L-Rhamnosidase was reportedly produced by some strains of human intestinal *Bacteroides* (Bokkenheuser *et al.* 1987; Jang and Kim 1996). These bacterial enzymes might play a major role in liberating the aglycones from sugar-conjugated soyasaponins in the human gut. The average length of time during which food residue stays in the large intestine is about 24 h or longer. Microbes in the large bowel would have sufficient time to interact with and hydrolyze soyasaponins. Further study will be needed to identify the bacterial species in the human intestinal tract that metabolize soyasaponins.

In our *in vitro* incubation study, soyasaponin III and soyasapogenol B were identified as the major microbial metabolites of soyasaponin I, implying their possible existence in the human gut. However, only a few studies investigated the biological activity of these soyasaponin metabolites to date. It has been known that the variety and the number of sugars attached to soyasapogenols have profound impact on their activity. Ikeda *et al.* (1998) found that soyasaponins with a disaccharide group (soyasaponin III and IV) were more potent in hepato-protective activity against immunologically-induced damaged than those with a trisaccharide group (soyasaponin I and II) and soyasapogenol B in the primary cultured rat

hepatocytes. Berhow *et al.* (2000) demonstrated that soyasapogenol B at non-cytotoxic levels was more potent than its various glycosides in suppression of 2-acetoxyacetylaminofluorene induced genotoxicity in Chinese Hamster Ovary cells. Rowlands *et al.* (2002) showed that soyasapogenol B inhibited the growth of human breast cancer cells *in vitro*. Therefore, the bioavailability and potential biological activities of these soyasaponin metabolites deserve further characterization due to their probable presence in the human lower intestinal tract.

The metabolism of soyasaponin I showed a biphasic pattern during 48 h incubation in the *in vitro* culture model (Figure 5). No significant degradation of soyasaponin I was observed during the first 4 h of incubation. Soyasaponin I content significantly decreased from 4 to 48 h in the culture system. The absence of soyasaponin I metabolism at the early stage of incubation may be because the microorganisms need time to adapt to the culture environment before they produce sufficient amount of enzymes to metabolize soyasaponins. Soyasaponin III was produced in the fecal incubations for 13 out of 15 subjects. Soyasapogenol B was produced in the fecal incubations for all the subjects.

The rate loss of soyasaponin I versus incubation time gave a linear relationship for all the subjects (Figure 6), indicating that disappearance of soyasaponin I follows an apparent first-order kinetics. The disappearance of soyasaponin I in the presence of human gut microorganisms varied among subjects. The average rate loss constant was $0.15 \pm 0.10 \text{ h}^{-1}$ and half-lives $t_{1/2}$ of soyasaponin I in the culture ranged from 2.2 to 19.1 h among the subjects. Two distinct degradation phenotypes were observed among the subjects according to the disappearance rate constants (Figure 5). The rate constants and half-lives of the two phenotypic groups were significantly different ($P < 0.05$): rapid soyasaponin degraders ($n=7$)

having a rate constant $k = 0.24 \pm 0.04 \text{ h}^{-1}$ and $t_{1/2} = 3.16 \pm 0.62 \text{ h}$; slow soyasaponin degraders ($n=8$) having $k = 0.07 \pm 0.02 \text{ h}^{-1}$ and $t_{1/2} = 11.15 \pm 4.34 \text{ h}$. The data demonstrate that the ability to metabolize soyasaponins by the gut microbes varied among our subjects and two soyasaponin metabolic phenotypes were present. If the ability to metabolize soyasaponins in the gut is a stable characteristic in humans, enhanced bioavailability and bioactivity of soyasaponins might be expected among the subjects exhibiting relatively longer soyasaponin half-life in the intestinal tract, whereas the subjects metabolizing soyasaponins rapidly would more likely experience effects of the metabolites.

The differences in the gut microflora populations and bacterial enzyme activities could lead to different microbial metabolism of soyasaponins. Our results demonstrate two distinguishable fecal soyasaponin metabolic phenotypes among our subjects. It might be because the two groups of subjects might have different gut microflora population and/or enzyme activities. There are many factors that influence gut microflora population and fecal enzyme activities, such as genetic background of individuals, dietary factors, physical activities and gut peristalsis (Rowland *et al.* 1988). Therefore, we examined the relationship of body mass index, stool moisture, gut transit time and soy consumption frequency with the ability of the subjects to metabolize soyasaponins by their gut microbes. The subjects' BMI ranged from 18.1 to 30.4 kg/m². The stool moisture was in a range of 47.2 to 78.3%. The gut transit time ranged from 38 to 168 h. There was no significant difference in body mass indices, gut transit time and stool moistures between two soyasaponin metabolic phenotypes (Table 2). According to their self-reported soy consumption, 5 subjects consumed soy foods more than once per week, 5 subjects consumed soy foods more than once a month but less

than once per week, and 5 consumed soy less than once a month or none. Soy consumption frequencies did not significantly affect soyasaponin metabolic rate among the subjects.

In our study, the subjects could be grouped into two ethnic populations: 5 Eastern Asians including 4 Chinese and 1 Indian, and 10 Caucasians. The distribution of subjects between two soyasaponin metabolic phenotypes was not significantly different between these two ethnic groups ($P = 0.07$). The Asian subjects showed a slightly higher soyasaponin I degradation rate with constant $k = 0.20 \pm 0.1 \text{ h}^{-1}$ than Caucasian subjects with the rate constant $k = 0.13 \pm 0.1 \text{ h}^{-1}$, but the difference was not statistically significant ($P = 0.18$). In our study, all Asian subjects were non-immigrants who came from eastern Asia and maintained their traditional diet when living in the United States. The Caucasian subjects were Americans who had a typical western diet. All the subjects in this study were omnivores. Zheng *et al.* (2001) reported that the subjects from eastern Asia who maintained their traditional diet apparently consumed more red meat and cholesterol than Caucasian subjects in their study. The subjects in our study were recruited from similar source compared to Zheng's study. Reddy *et al.* (1973) found that the total anaerobic microflora such as *Bacteroides*, *Bifidobacteria*, *Peptococci* and *Lactobacilli* were significant higher in the feces after the subjects were shifted from non-meat diet to high-meat diet. These strains of bacteria have been found to possess glycosidase and/or β -glucuronidase activities (Rowland *et al.* 1970). High meat diets have been associated with decreased fecal bacterial β -glycosidase activity and increased enzyme activities including β -glucuronidase, nitroreductase and azo-reductase (Reddy *et al.* 1974; Goldin *et al.* 1978). Therefore, we expected that the Asian subjects would have higher soyasaponin degradation rate than Caucasians since the diet high in red meat and cholesterol consumed by the Asian subjects

might induce higher β -glucuronidase activities in their gut so that soyasaponins were metabolized to their aglycones more rapidly. No significant difference in soyasaponin degradation rate we observed in this study could be due to the small sample size that limited statistical power of the analysis. In order to further elucidate the influence of ethnic and dietary factors on soyasaponin metabolism, the study with larger sample size will be necessary.

Our present study may provide insight in the role of gut microflora on soyasaponin bioavailability and biological potency. The potential of soyasaponins to lower blood cholesterol has been proposed. Soyasaponins could form insoluble complexes with cholesterol and interfere with enterohepatic circulation of bile acids by forming mixed micelles, and inhibit the intestinal absorption of endogenous and exogenous cholesterol (Oakenfull *et al.* 1990). Soyasaponins have been shown anticarcinogenic effects on colon cancer in animal model (Korotkar *et al.* 1997). This colon cancer inhibiting activity of soyasaponins may be contributed by their antimutagenic, cytotoxic and differentiation-inductive effects on cancer cells (Sung *et al.* 1995; Berhow *et al.* 2000; Oh & Sung 2001). In addition, the interaction between soyasaponins and bile acids to reduce the formation of secondary bile acids in the gut might also contribute to soyasaponin's colon cancer suppressing activity. Thus, slow soyasaponin degraders would be able to retain original soyasaponins longer in the gut to interact with cholesterol and bile acids, therefore have greater cholesterol-lowering and colon cancer inhibiting effects from ingested soyasaponins. Although the role of soyasapogenols in the health beneficial effects of soyasaponins is unknown, rapid soyasaponin degraders might have higher concentrations of soyasapogenols

in the gut, thus experience a greater bioavailability and impact from the soyasaponin aglycones.

The data from this study support that soyasaponin I can be metabolized by human gut microorganisms to the molecules with fewer or no sugars attached to the aglycone. The reaction may be enzymatic in nature. It is reasonable to infer that other forms of soyasaponins may be metabolized in the gut in the same manner. Two distinguishable soyasaponin metabolic phenotypes, slow soyasaponin degraders and rapid soyasaponin degraders, were observed among human subjects in our study, implying that different people might experience different spectrum of biological effects from soyasaponin ingestion. Individual variation in soyasaponin metabolism by gut microflora may be present in humans. This variation might be due to the difference in the gut microflora population and/or bacterial enzyme activity, which might be affected by the subjects' genetic background, dietary habits and gut mobility. It would be of importance to further characterize the factors that influence individual ability to metabolize soyasaponins in the gut. In addition, the study on the bioavailability and biological activities of soyasaponins and their gut metabolites would be essential to evaluate the health-promoting role of dietary soyasaponins in the future.

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Legends to the Figures:

Scheme 1. Structures of soyasaponin I and its metabolites.

Figure 1. Ion chromatograms of soyasaponin I (A), soyasaponin III (B) and soyasapogenol B (C) standards with a positive mode ApCI- MS detection. The chromatogram of 12 and 48 h fecal incubation extracts from one subject are shown in D and E, respectively.

Figure 2. Positive mode ApCI mass spectra of soyasaponin I (A), soyasaponin III (B) and soyasapogenol B (C).

Figure 3. Silica TLC profile of soyasaponin I degradation in the fecal fermentation system. A 10 μ L sample from each fecal extract was spotted on the plate. The plate was developed with butanol-ethanol-ammonia hydroxide (5:5:4).

Figure 4. Comparison of *in vitro* metabolism of soyasaponin I in fecal fermentation system among different experimental treatments. Each bar represents the value of mean \pm SD of three replicates.

Figure 5. *In vitro* metabolism of soyasaponin I in fecal fermentation system measured by HPLC. Disappearance of soyasaponin I and appearance of its metabolites I and II was monitored at UV 205 nm. Each point represents the value of mean \pm SD of three replicates.

Figure 6. Rate loss of soyasaponin I versus incubation time from all the subjects (n = 15). Different symbols represent different subjects.

Table 1. TLC, HPLC and mass spectral data of soyasaponin I, III and soyasapogenol B

	Molecular weight *	TLC Rf	HPLC retention time	APCI-Mass Spec (positive mode)
Soyasaponin I C₄₈H₈₀O₁₈	944	0.60 (butanol-ethanol-ammonia)	25.9 min by program 1	943.3 [M+H] ⁺ , 797.4 [M-rhm+H] ⁺ , 599.4 [M-glc-gal-2H ₂ O+H] ⁺ , 441.3 [aglycone-OH] ⁺ , 423.4 [aglycone-OH-H ₂ O] ⁺ , 405 [aglycone-OH -H ₂ O] ⁺ on APcI
Soyasaponin III C₄₂H₆₈O₁₄	796	0.63 (butanol-ethanol-ammonia)	29.9 min by program 1	797.3 [M+H] ⁺ , 599.4 [M-rhm-gal-2H ₂ O+H] ⁺ , 441.3 [aglycone-OH] ⁺ , 423.3 [aglycone-OH-H ₂ O] ⁺ , 405.3 [aglycone-OH -H ₂ O] ⁺ on APcI
Soyasapogenol B C₃₀H₅₀O₃	458	0.43 (hexane:ethyl acetate)	27.2 min by program 2	441.3 [aglycone-OH] ⁺ , 423.3 [aglycone-OH-H ₂ O] ⁺ , 405.4 [aglycone-OH -H ₂ O] ⁺ on APcI

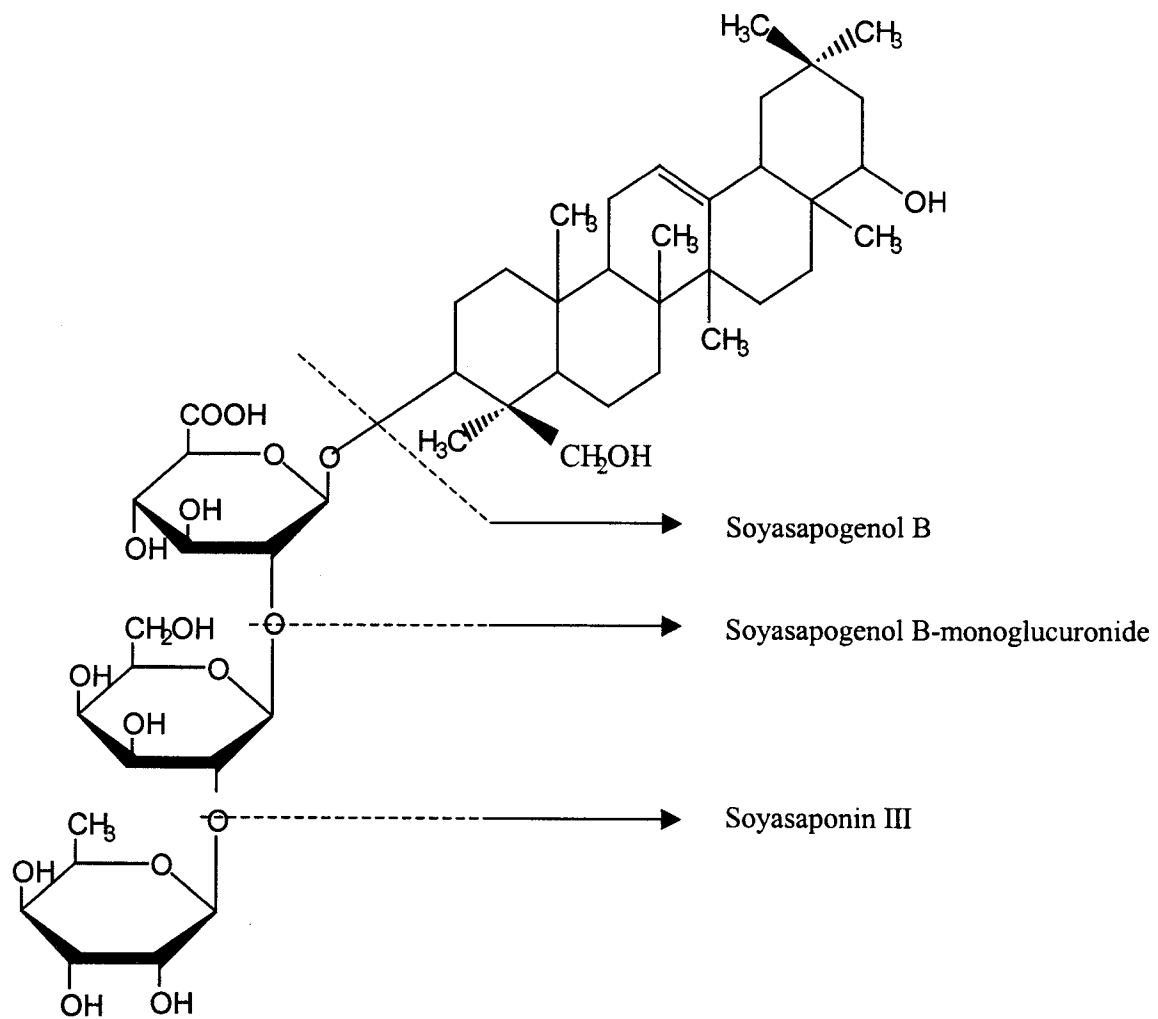
* The molecular weight is calculated value based on the molecule formula.

Table 2. Comparison of body mass index (BMI), gut transit time (GGT), stool moistures, ethnicity distribution and rate loss of soyasaponin I between two soyasaponin metabolic phenotypes

Phenotype	Rate constant (h ⁻¹)	Half-life (h)	BMI (kg/m ²)	GGT (h)	Stool moisture (%)	Ethnicity distribution (Asian/Caucasian)
Slow (n = 8)	0.07 ± 0.02 ^a	3.16 ± 0.62 ^a	24.4 ± 7.3	130.6 ± 40.7	60.3 ± 5.3	1 / 7
Rapid (n = 7)	0.24 ± 0.05 ^b	11.15 ± 4.34 ^b	21.4 ± 3.4	69.7 ± 38.2	65.3 ± 10.4	4 / 3

Values are expressed as MEAN ± SD. The phenotype was distinguished by cluster analysis. The values in the same column with different superscripts are significantly different (P < 0.05).

Scheme 1



Soyasaponin I

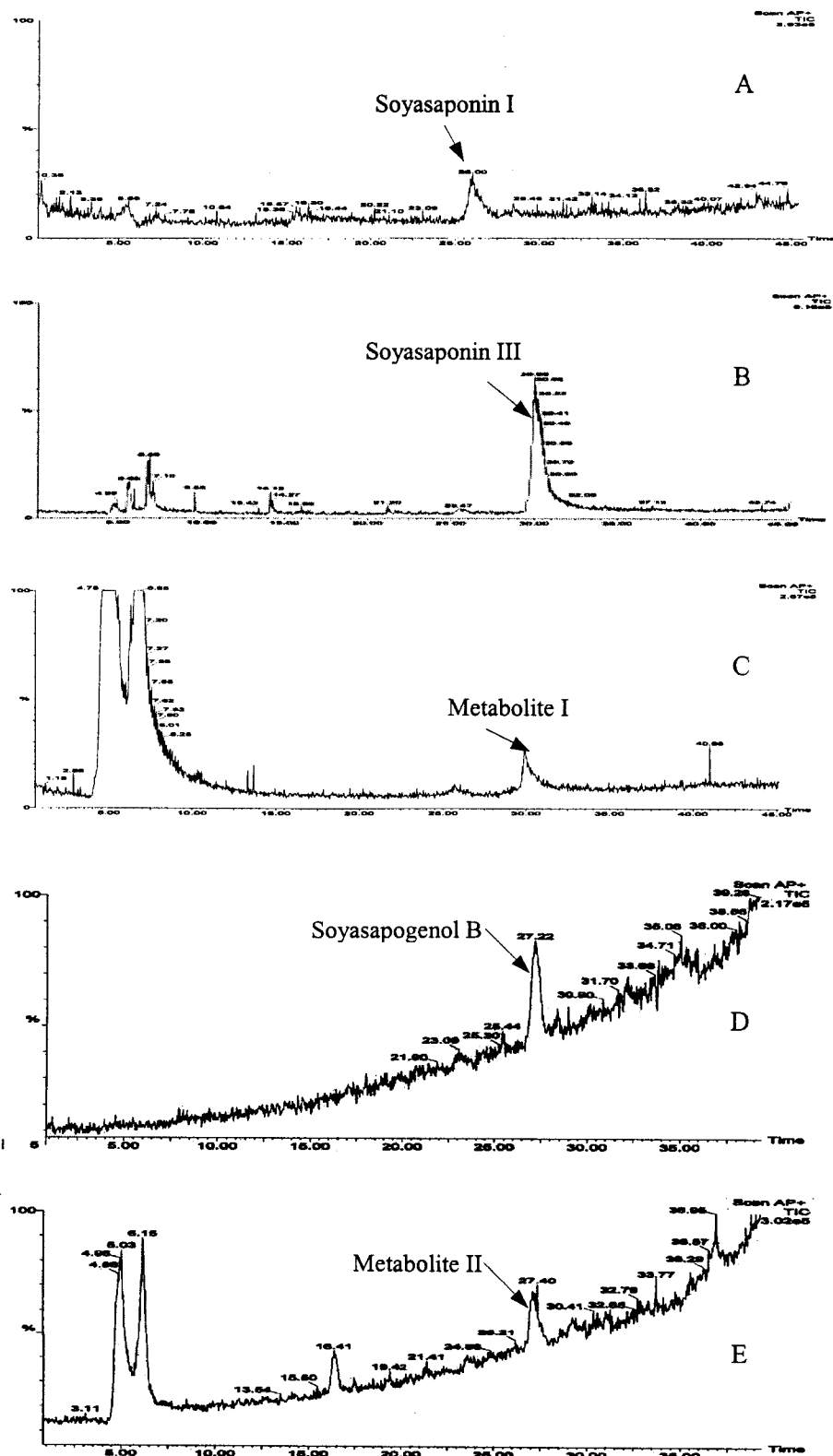


Figure 1

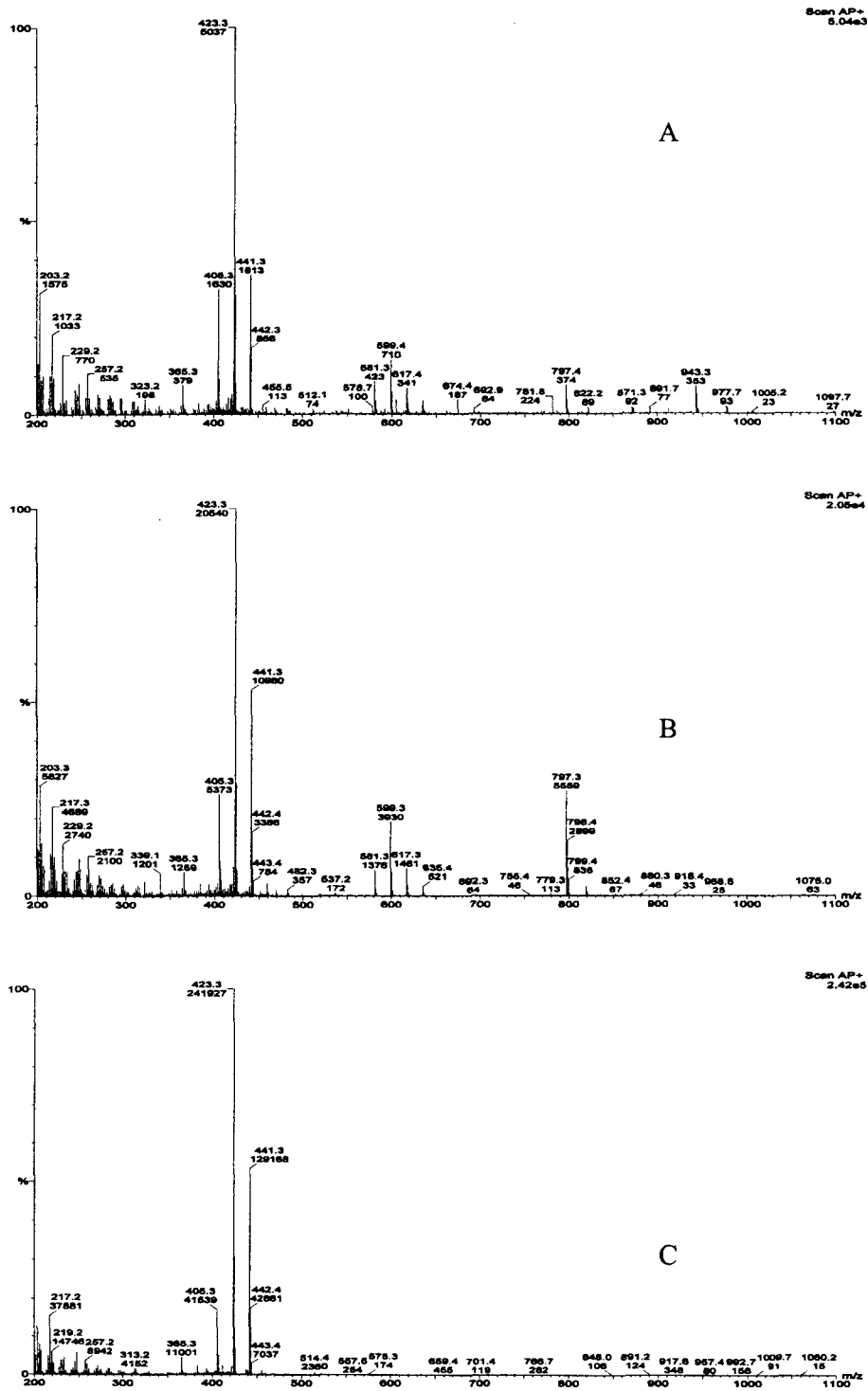
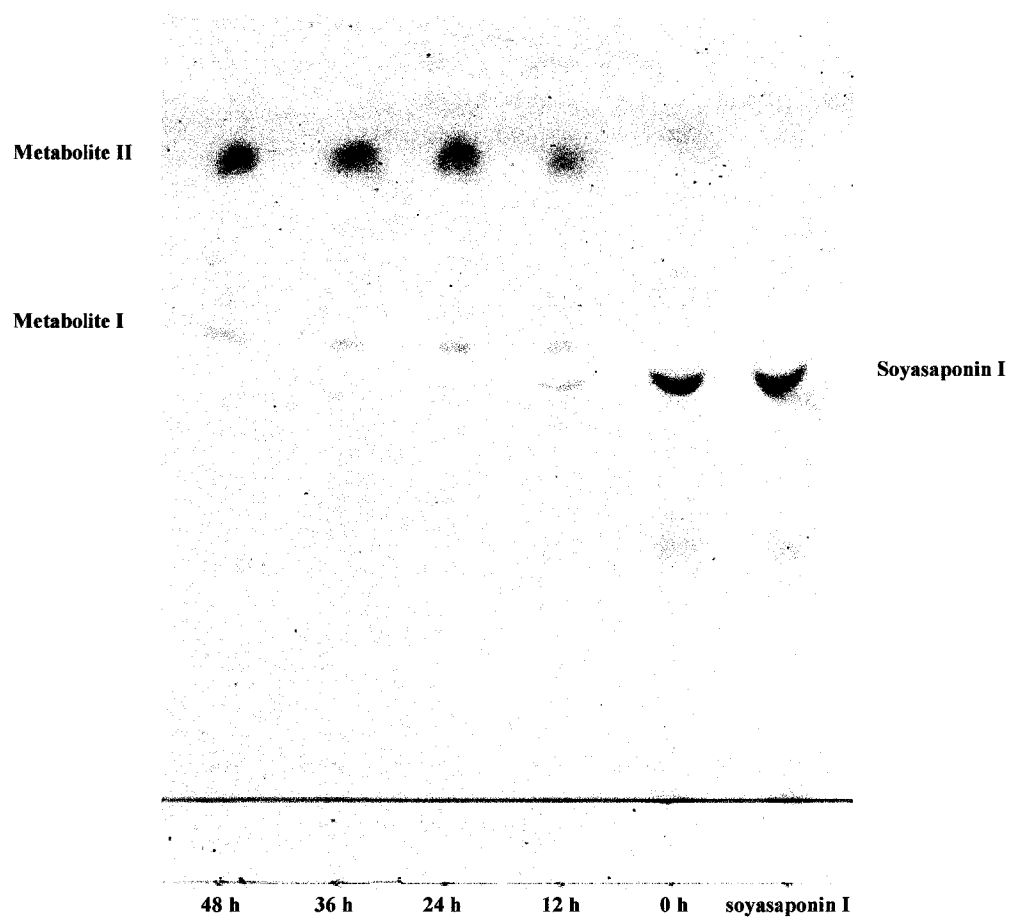
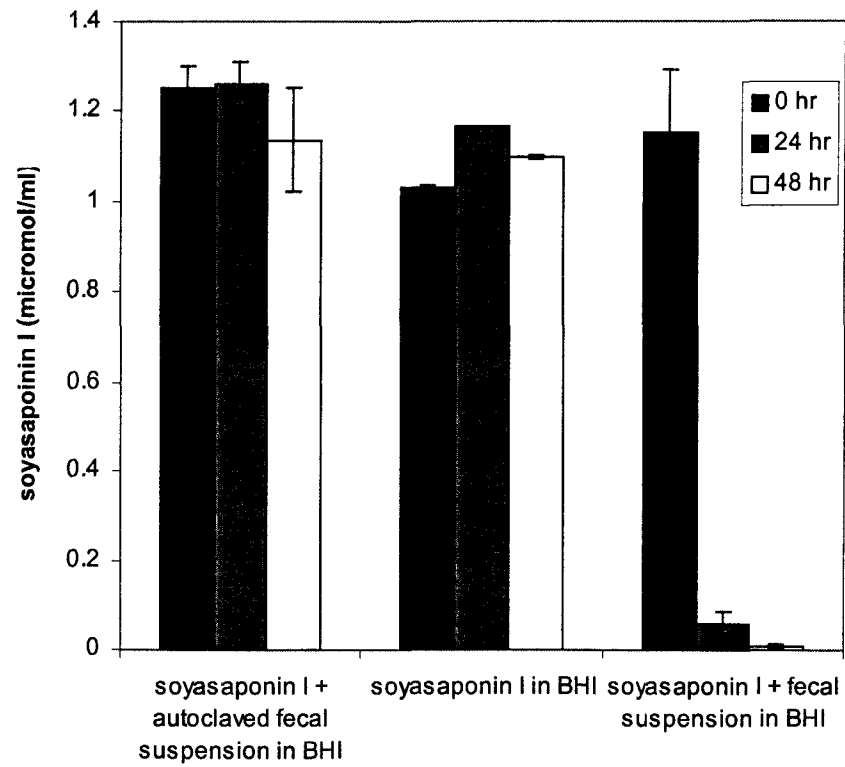
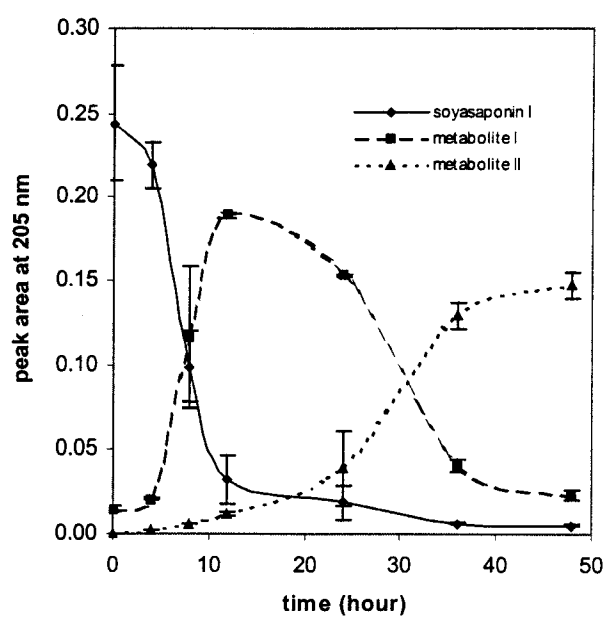


Figure 2

**Figure 3**

**Figure 4**

**Figure 5**

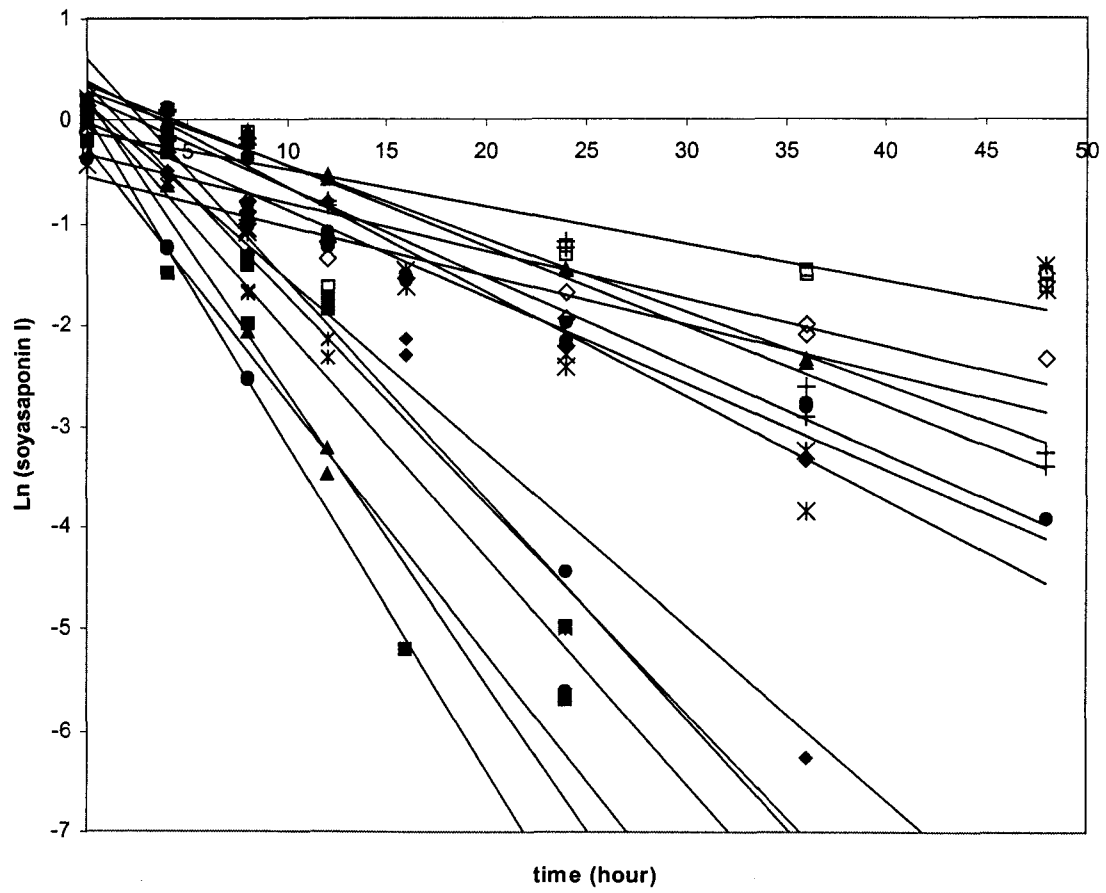


Figure 6

HUMAN INTESTINAL ABSORPTION AND BIOAVAILABILITY OF SOYASAPONIN I

A paper to be submitted to the Journal of Nutrition

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ABSTRACT

A human study was conducted to evaluate dietary soyasaponin bioavailability *in vivo*. Eight healthy women ingested a single dose of concentrated soy extract containing 434 μ moles of group B soyasaponins, the dominant forms of soyasaponins in soybeans. No soyasaponins or their gut metabolites were detected in 24 h urine. Soyasapogenol B, a major gut metabolites of group B soyasaponins, was detected at a total of 36.6 ± 16.9 μ moles in a 5-day feces collection but no group B soyasaponins was detected in feces. The data showed that ingested group B soyasaponins were metabolized to soyasapogenol B in the human intestine and excreted in the feces. A human colon cancer Caco-2 cell transfer model was used to evaluate absorbability and transport kinetics of soyasaponins at the cellular level. In the Caco-2 cell transfer model, 0.5 to 2.9% and 0.2 to 0.8% of soyasaponin I and soyasapogenol B dosed in the apical chamber, respectively, appeared at the basolateral side after 4 h incubation. The apical-to-basolateral absorption of soyasaponin I and soyasapogenol B was low with P_{app} of 0.9 to 3.6×10^{-6} cm/sec and 0.3 to 0.6×10^{-6} cm/sec, respectively. The transport rate and uptake of soyasaponin I by Caco-2 cells appeared to be saturable and concentration-independent. In contrast, soyasapogenol B accumulated in the Caco-2 cells in a concentration-dependent manner. The cytotoxicity assay showed that

soyasaponin I had no apparent cytotoxic effect on Caco-2 cells at concentrations up to 3 mM, while soyasapogenol B at 1 mM or higher concentration significantly reduced cell viability.

Keywords: soyasaponin I, soyasapogenol B, Caco-2 cell monolayer

INTRODUCTION

Soyasaponins are one of the primary phytochemicals present in leguminous seeds (Price *et al.* 1986; Tsukamoto *et al.* 1995). The basic structure of soyasaponins is an oleanene-type triterpenoid aglycone with one or more polysaccharide chains attached, resulting in an amphiphilic nature of the molecules. Soyasaponins in soybean seeds and various soy products become the primary source of saponins from foods due to increasing soy consumption in Western countries. Significant amounts of soyasaponins are found in soybeans and soy products in a concentration range generally between 0.5 to 114 $\mu\text{mol/g}$ (Hu *et al.* 2002). Soyasaponins have drawn great interest in recent years because these compounds have been demonstrated to possess multiple health-promoting properties, such as plasma cholesterol-lowering (Oakenfull *et al.* 1990; Potter *et al.* 1995), anticarcinogenic (Rao *et al.* 1995; Konoshima *et al.* 1996), hepatoprotective (Miyao *et al.* 1998; Kinjo *et al.* 1998), and anti-viral activities (Nakashima *et al.* 1989; Hayashi *et al.* 1997). Most of the studies investigating soyasaponin biological activities have been limited to *in vitro* experiments and a few animal studies. The relevance of these results to humans under *in vivo* conditions is not clear since little is known about how and to what extent dietary soyasaponins may enter systemic circulation after ingestion.

There is very limited knowledge on the bioavailability of soyasaponins and soyasapogenols. Saponins have been assumed to be poorly absorbed in the intestine. It was thought that sugar

chains of saponins had to be hydrolyzed to liberate the aglycones by the bacterial enzymes in the lower intestine. The metabolism of soyasaponins by gut microbes was supported by the data from Gestetner *et al.* (1968) and Hu *et al.* (unpublished data 2003). They both demonstrated that soyasaponins could be metabolized by intestinal microflora to release sugars and aglycones as the metabolites in animals and humans. Soyasaponin aglycones, named soyasapogenols, have been shown to be more effective than its various glycosides in suppression of 2-acetoxyacetylaminofluorene induced genotoxicity on Chinese Hamster Ovary cells (CHO) (Berhow *et al.* 2002). Rowlands *et al.* (2002) suggested that soyasapogenol B had growth inhibitory effect on human breast cancer cells.

Gestetner *et al.* (1968) examined soyasaponin absorption in rats, chickens and mice after oral dosing the animals. In their study, soyasapogenols and soyasaponins were determined qualitatively in the digestive tract and blood samples using TLC and hemolysis analysis. Neither soyasaponins nor soyasapogenols were found in the urine or blood of the animals, suggesting that dietary soyasaponins might not be absorbed in these animals. However, there is no direct evidence to demonstrate the absorbability and pharmacokinetics of soyasaponins in humans.

The present study focused on investigating the bioavailability of soyasaponins and soyasapogenols in humans. Soyasaponin I (Scheme 1), as a representative of soyasaponins, was examined in the study because it is a principal form of soyasaponins in heat-treated soy products (Hu *et al.* 2002; Gu *et al.* 2002). Soyasapogenol B, a major gut microbial metabolite of soyasaponin I, was used to evaluate absorbability of soyasaponin aglycones. A single dose human feeding study was conducted to evaluate dietary soyasaponin bioavailability *in vivo*. The transepithelial absorbability and transport kinetics of these

compounds were evaluated using the human colon carcinoma Caco-2 cells, a well-established human intestinal absorption model. The results obtained from this study will help to understand bioavailability of dietary soyasaponins and the absorption of their gut metabolites in humans, and to predict their potential beneficial effects.

MATERIALS AND METHODS

Preparation of soyasaponin I and soyasapogenol B standards. Soyasaponin I was isolated from soy germ supplied by Schouten USA, Inc following Hu's method (Hu *et al.* 2002). Soyasapogenol B was produced by hydrolyzing 100 mg of group B soyasaponins in 3 *N* hydrochloric acid refluxing for 3 h. Group B soyasaponins mixture was generously provided by Dr. Mark Berhow (National Center for Agricultural Utilization Research, USDA). The reaction mixture was neutralized with 10 *N* sodium hydroxide and desalted with a high capacity C₁₈ SPE column (Alltech Associates Inc., Deerfield, IL) with sequential elution of water and methanol. Crude soyasapogenol B was obtained in the methanol fraction. Twenty-four milligrams of soyasapogenol B was harvest after further purification with a semi-preparative HPLC system (Hu *et al.* 2002) using 70 % aqueous acetonitrile with 1 *mM* ammonium acetate at a flow rate of 2 mL/min. The identity of purified soyasaponin standards was confirmed by electrospray ionized (ESI) mass spectroscopy.

Human feeding study. The subjects were 8 healthy, non-smoking women aged 25 to 34 years, with a body mass index of 21.0 ± 2.6 kg/m², and without taking any medication for 3 months prior to and during the study. The study protocol was reviewed and approved by the Iowa State University Human Subjects in Research Committee in 2001. Informed

consent was obtained from each subject before the experiment was initiated. The subjects were instructed to avoid soyasaponin-containing foods (Appendix 1) for 4 d as a soyasaponin wash-out period. At the end of the wash-out period, the subjects ingested 4 g of Prevastein® (Central Soya Company, Inc., Fort Wayne, IN), a concentrated soy extract containing 108.9 μ moles group B soyasaponins /g, at breakfast after overnight fasting. The subjects took a carmine red dye marker capsule (500 mg, University of Iowa, Iowa City, IA) as the indicator of gut transit time with the soy dose. A baseline urine sample was collected from each subject before dosing, and a 24 h urine was collected after dosing. The total volume of urine was recorded for each subject. A 50.0 mL sample was taken from the urine and freeze-dried. All the stools were collected from the subjects from the time of dosing until the red dye marker disappeared in the feces. Fecal samples were freeze-dried. All the samples were stored at - 20 °C until analyzed.

The freeze-dried urine sample was dissolved in 25 mL of 0.2 *M* sodium acetate buffer at pH 5.5 with or without 100 μ L of β -glucuronidase/sulfatase (86,900 units/mL, Sigma-Aldrich, St. Louis, MO). The mixture was incubated at 37 °C for 8 h. The enzyme treatment was used to hydrolyze any possible UDP-conjugated or sulfate-conjugated forms of soyasaponin or soyasapogenols. After incubation, the sample was applied onto a pre-conditioned Sep-Pak cartridge (classic short-body C₁₈, Waters Corp. Milford, MA). The cartridge was washed with 5 mL of distilled water followed by 5 mL of 5% methanol. Soyasaponins were eluted with 2.0 mL HPLC grade methanol. The extract was vortexed and filtered through a 0.45 μ m PTFE filter (Alltech Associates Inc., Deerfield, IL) prior to thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) analyses.

Three grams of freeze-dried fecal sample was weighed and extracted with 100 mL of 70% ethanol at room temperature (RT) for 2 h. The extract was filtered through Whatman No. 42 filter paper. The filtrate was evaporated to dryness at RT using a rotary evaporator at reduced pressure. The residue was suspended in 5 mL of 20% methanol and loaded onto a pre-conditioned Sep-Pak cartridge. The cartridge was washed with 5 mL of distilled water followed by 5 mL of 30% methanol. Soyasaponins were eluted with 3.0 mL HPLC grade methanol. The extract was vortexed and filtered through a 0.45 μ m PTFE filter prior to TLC and HPLC analysis. All the urine and fecal samples collected were extracted and analyzed in duplicate.

TLC analyses were performed on silica gel LK6F plates (Whatman, Hillsboro, OR). The urine and fecal samples for each subject were analyzed under two different conditions: one was developed with butanol-ethanol-ammonia (5:5:4, v/v); the other was developed with hexane-ethyl acetate (2:1, v/v). Soyasaponins were detected by spraying acetic acid-sulfuric acid-anisaldehyde (100:2:1, v/v) and heating at 120 °C for 10 min. Soyasaponins and soyasapogenol B displayed a blue-purple color on the TLC plate.

Soyasaponin I concentration was determined by HPLC analysis as previously reported (Hu *et al.* 2002). The mobile phases were 0.05% trifluoroacetic acid in water (solvent A) and acetonitrile (solvent B). The gradient elution was carried out as follows: solvent B held at 37% for 3 min, then increased from 37 to 40% in 12 min, then solvent B increased to 48% in 25 min, and finally solvent B increased to 100% in 1 min and remained at 100% for 2 min. The gradient program recycled back to the initial state of 37% solvent B in 5 min. The column temperature was 30 °C. The injection volume was 50 μ L. The flow rate was 1 mL/min, and the UV absorbance was monitored from 190 to 350 nm.

Soyasapogenol B concentration was measured with the same HPLC system using a different gradient program: solvent B increased from 73 to 100% linearly in 35 min, then solvent B recycled back to 73% in 4 min.

Transepithelial absorption and transport kinetics of soyasaponin in the Caco-2 cell model. Caco-2 cells were purchased at passage 18 from American Type Culture Collection (Rockville, MD). The experiments were conducted at passage 35-45. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Sigma, St. Louis, MO) with 16% fetal bovine serum (Sigma, St. Louis, MO), 1% non-essential amino acids (Gibco BRL, Grand Island, NY) and 1% antibiotic-antimycotic solution (Gibco BRL) at 37°C in an incubator with 5% CO₂/ 95% air. Cells were seeded on the collagen-coated polytetrafluoroethylene membrane inserts (0.45 µm, 1 cm² surface area) with a density of 5.5 × 10⁴/cm² for the transport experiments. The inserts were fitted in 6-well bicameral chambers (Transwell-COL, 24 mm ID, Corning Costar Corp., Cambridge, MA) and cultured for 14 to 16 days until 95 to 100% confluence was reached and cells became well differentiated. Phenol red test (Garcia *et al.* 1996) was performed to measure cell monolayer integrity before and after the transport assay. A serum-free medium was used as the transport buffer to perform transport assay of soyasaponin across Caco-2 cell monolayer. The serum-free medium contained 1% antibiotic-antimycotic solution, 4 mg/L hydrocortisone, 10 mmol/L PIPES, 5 µg/L selenium, and 34 µg/L T₃ in DMEM medium.

Transepithelial transport was measured for soyasaponin I and soyasapogenol B at concentrations of 0.5, 1 and 3 mM. The transport assay for each compound at each concentration was done in triplicate. Soyasaponin I or soyasapogenol B was suspended in the transport buffer and sonicated for 30 s with a sonic demembrator (Fisher Scientific,

Pittsburgh, PA). Caco-2 monolayers grown on the membrane inserts were first rinsed with 2 mL of Earl's Balanced Salt Solution (EBSS) buffer and then bathed in 2 mL of transport buffer at 37 °C for 15 min prior to the treatment. Then the apical buffer was replaced with 1.5 mL of transport buffer containing the test compound. A 1.0 mL volume of transport buffer without the test compound was added to the basal chamber. The system was incubated at 37°C for 4 h. The samples were taken from the basal chamber at 30 min, 1, 2, and 4 h intervals. This was accomplished by replacing the basal chamber buffer with 1.0 mL of fresh transport buffer at each time point. Cumulative transport rates were then determined by summing the amount of the compound transported to the basal chamber from the discrete time points (Coghurn *et al.* 1991). At the end of experiment, the buffer in the apical chamber was collected to determine the remaining amount of the test compound. All the samples collected were stored at - 20 °C until analysis.

The amount of test compound on the insert was measured as well to determine its cellular uptake. After the transport assay, the apical and basal chambers were rinsed 3 times with 1 mL of ice-cold EBSS buffer. The membrane inserts were then peeled off from the membrane holders and placed in 1.5 mL of ice-cold 0.5 M sodium hydroxide to solublize the cells. The cells were further lysed by sonicating with the sonic demembrator for 30 s (Au and Reddy 1999). The total protein content of cells on the inserts was determined using Lowry's method (Lowry *et al.* 1951) to ensure there was comparable numbers of cells on each insert used in the experiments.

The contents of soyasaponin I or soyasapogenol B in the samples were determined as follows. The collected sample was thawed to RT. The sample from the basal chamber was directly loaded onto a preconditioned Sep-Pak cartridge (Light short-body C₁₈, Waters Corp.

Milford, MA). The cartridge was washed with 3 mL of 5% methanol. Soyasaponin or soyasapogenol B was then eluted with 0.8 mL HPLC grade methanol. The sample from the apical chamber was loaded onto a larger size pre-conditioned Sep-Pak cartridge (Classic short-body C₁₈). Soyasaponin or soyasapogenol B was eluted with 2.0 mL HPLC grade methanol. The extract was vortexed and subjected to HPLC analysis. The defined permeability coefficients (P_{trans}) were determined using the following equations (Artursson 1990; Cogburn *et al.* 1991; Oitate *et al.* 2001):

$$P_{app} = \Delta Q / (\Delta t \times 60 \times A \times C_0)$$

$$P_{trans} = 1/P_{app} - 1/P_{filter}$$

where P_{app} and P_{filter} are the apparent permeability coefficients estimated by transport assay in the presence and absence of Caco-2 cells, respectively. $\Delta Q/\Delta t$ is the permeability rate constant ($\mu\text{mole}/\text{min}$), A is the surface area of the membrane (cm^2), and C_0 is the initial concentration of the compound in the apical chamber ($\mu\text{mole}/\text{mL}$). All rate constants were obtained as the slopes of the regression line for receiving amount in the basal chambers versus time. The transport assay was done under sink conditions, i.e., experiment was done before > 10% of the compound had been transported to the basal chamber.

The acute toxicity of soyasaponin I and soyasapogenol B to Caco-2 cells was evaluated at the concentrations used in the transport assay. Caco-2 cell monolayers were treated with the transport buffers containing soyasaponin I or soyasapogenol B as treatment groups. The control was treated with the transport buffer without test compounds. Each treatment was done in duplicate. After 4 h incubation, the insert was rinsed with 1.5 mL of 1 M phosphate buffer. The cells were digested with 0.5 mL of trypsin-EDTA and suspended in

the serum-free medium. The viability of the harvested cells was determined with trypan blue dye exclusion method (Butler and Dawson 1992).

Statistical analysis. All data were expressed as mean \pm SD. Statistical analyses were performed with SAS system (Version 8.1, SAS Institute Inc., Cary, NC). The transport kinetics of soyasaponin I and soyasapogenol B across the Caco-2 cell monolayer was analyzed by general linear regression. The differences in cell uptake and cytotoxicity of soyasaponin I and soyasapogenol B at different concentrations were compared using analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The difference was considered to be significant when the P value was less than 0.05.

RESULTS AND DISCUSSION

In order to evaluate the bioavailability of dietary soyasaponins, we first conducted a human study with a single oral administration of soy concentrate. This single dose of soy concentrate contained 435.9 μ moles of total group B soyasaponins. The excretion of soyasaponins or their gut metabolites in the urine and feces was monitored after dosing, and the results are shown in Table 1. No group B soyasaponins or soyasapogenol B was detected in the 24 h urine regardless of whether or not the sample was treated with β -glucuronidase/sulfatase during sample extraction. Soyasapogenol B was detected in the collected feces, but soyasaponins were not found. The total amount of soyasapogenol B recovered in the feces was 36.3 ± 3.6 μ moles (Mean \pm SE). More than 65% of detected soyasapogenol B was excreted in 1 to 3 days after dosing. The total soyasapogenol B recovered from the feces was 8.4% of the total ingested group B soyasaponins on a mole basis. Variation in soyasapogenol B recovered from the feces was different among subjects,

especially during Day 0 and Day 1. Three of the subjects excreted a small amount of soyasapogenol B on Day 0, the day that soy dose was given. It is likely that the foods which the subjects consumed during wash-out period might contained soyasaponins but not been known to us.

Gestetner *et al.* (1968) examined the fate of soyasaponins in different animal models. Neither soyasaponin nor soyasapogenol was detected in the blood after orally dosing the animals with soyasaponins, whereas soyasapogenols were recovered from the cecum and colon of the animals. Hu *et al.* (2003) observed that group B soyasaponins were converted into their aglycone by human intestinal microflora *in vitro*, suggesting that the aglycone of group B soyasaponins, soyasapogenol B, might be a potential gut metabolite of dietary soyasaponins in humans. Our data implies that ingested group B soyasaponins can be metabolized to soyasapogenol B in the human intestine *in vivo*.

In this study, only 8.4% of ingested group B soyasaponins was recovered as their aglycone form, soyasapogenol B, in the feces. In addition, soyasaponins and soyasapogenol B were absent in the urine. In Gestetner's study, 60 to 65% of total ingested soyasaponins was recovered as the form of soyasapogenols in the collected feces of rats. The recovery in our study is much lower than their findings in animals. We propose two possibilities that might contribute to this phenomenon. One possibility is that soyasaponins might undergo biotransformation by gastrointestinal mucosa and the liver before entering the systemic circulation. Since no soyasaponins or soyasapogenol B was found in the urine treated with β -glucuronidase/sulfatase, soyasaponins or soyasapogenol B *in vivo* might be metabolized by phase I transformation enzymes, if it occurs, which would result in the alteration of the aglycone structure. The other possibility is that soyasaponins and soyasapogenol B might not

be absorbed in the intestine but further metabolized by gut microflora to smaller degradation products and excreted in the feces. These degradation products would not have been recognized by the analytical methods we used. It would be valuable for the future work to have soyasaponins with a stable isotope labeled aglycone to trace the fate of ingested soyasaponins in animal models or humans.

Very limited information is available on the bioavailability of other types of saponins. Glycyrrhizin, a triterpene saponin found in licorice, has been reported to be metabolized by human intestinal microflora into the aglycone, glycyrrhetic acid (Kim *et al.* 2000). Glycyrrhetic acid was absorbed and displayed an anti-inflammatory activity in mice (Horigome *et al.* 2001). Hasegawa *et al.* (1996) reported that metabolite I of ginseng saponins was detected in blood at a concentration of 0.2 µg/mL after an oral administration of 150 mg ginseng /kg body weight to a human subject. The original ginsenosides and their metabolites were detected in the blood and urine after single oral administration of 1g ginsenosides /kg body weight in rats. However, the doses used in their study were so high compared to dietary levels that the permeability of intestinal mucosal cells might have been increased (Johnson *et al.* 1986). It is likely that the observed ginseng saponins and their metabolites in urine might be leaked from the intestinal tract instead of being absorbed by enterocytes.

For comparison, the gastrointestinal absorption of soy sterol and soy stanols in humans is very low (Ostlund *et al.* 2000). After a single meal of 600 mg lecithin-emulsified soy stanols or sterols, the absorption of sitosterol, campesterol, sitostanol, and campestanol was only 0.51%, 2.2 %, 0.044% and 0.26%, respectively. Soyasaponins are larger molecules than these phytosterols but have a similar hydrophobicity. It is likely that soyasaponins

might have very low oral bioavailability that is similar to phytosterols. Klaassen and Rozman (1996) suggested that bioavailable substances should not only be absorbed, but also survive the first-pass effect, which refers to biotransformation by the gastrointestinal cells or the liver and excretion into the bile with or without prior biotransformation. Urinary and plasma recovery therefore serve as reasonable indicator for bioavailability. Soyasaponins or soyasapogenol B might not be truly bioavailable based on this definition. However, the health-protective effects of substances also depend on their active forms, which could be the parent compounds or their metabolites produced *in vivo*. Failing to observe the parent compounds in the urine after oral dosing may not necessarily indicate their limited health beneficial effects. More research needs to be done in order to understand the mechanism of pre-systemic metabolism and first-pass elimination of soyasaponins in humans.

The absorbability and transport kinetics of soyasaponin I and soyasapogenol B were evaluated at the cellular level using a Caco-2 cell monolayer model to estimate their intestinal bioavailability. The concentrations used in this study were based on the dose of group B soyasaponins given in the human study above, i.e. 436 $\mu\text{moles/person}$. Assuming soyasaponins and their gut microbial metabolite, soyasapogenol B, stayed in the lower intestine unabsorbed, and an average 150 to 300 g/day of stool, the resulting concentration of soyasaponins or soyasapogenol B would be 1.5 to 3 $\mu\text{mole/g feces}$. Thus, the concentration range of 0.5 to 3mM was used considering the reported toxicity of higher doses of soyasaponins to the cultured colon carcinoma cells (Sung *et al.* 1995).

In our study, the total protein content of Caco-2 cell monolayers used in the experiment was $1210.9 \pm 162.3 \mu\text{g/insert}$. This suggests that the number of cells per insert was comparable among individual inserts used in the experiment. The integrity of Caco-2

cell monolayers was maintained well with confluence above 92% during the experiment. Chao *et al.* (1998) observed that 0.01 to 0.1% of quillaja saponin DS-1 in the culture medium increased the permeability of mannitol and d-decapeptide over Caco-2 cell monolayer without causing any detectable morphological changes of the monolayer. However, in our study, applying soyasaponin I and soyasapogenol B up to 0.3% (w/v) in the apical chamber apparently did not affect the monolayer confluence significantly. Our data suggest that soyasaponins might be less effective on disturbing the integrity of Caco-2 cell monolayer. This is also supported by Johnson's (1986) observation that soyasaponins were less capable of increasing the permeability of rat intestinal mucosal cells *in vitro* in comparison with gypsophylla saponins and saponaria saponins.

The recoveries of soyasaponin I and soyasapogenol B were calculated based on the amount of compounds recovered in both the apical and basal chambers as well as the cell insert after the 4 h transport assay. The total recovery was $101.3 \pm 3.7\%$ and $76.7 \pm 6.5\%$ for soyasaponin I and soyasapogenol B, respectively. The recovery was significantly greater for the glycoside, soyasaponin I, than the aglycone, soyasapogenol B. Our sample extraction efficiencies for soyasaponin I and soyasapogenol B analyses were all above 92%. There are two possibilities that might explain the 25% loss of soyasapogenol B on its mass balance. It is possible that highly hydrophobic soyasapogenol B might be adsorbed to the surface of polystyrene chambers during the incubation. In our study, a consistent percentage of the recovery on soyasapogenol B was observed among the three different concentrations. With the same volume of solution in the chambers, the more concentrated the solution was, the more soyasapogenol B molecules would interact with the chamber side-wall and be adsorbed to its surface, provided that the surface area was not saturated at the highest concentration of

soyasapogenol B. Artursson (1990) demonstrated that the insert membrane made of cellulose esters adsorbed significant amounts of tested drugs compared to the polycarbonate membrane. This effect appeared to be more significant for the drugs with higher hydrophobicity. We are not aware of any evidence in the literature regarding the affinity of hydrophobic compounds to polystyrene culture wells. It would be important to understand the relationship of compound hydrophobicity with its affinity to the culture well surface in the future studies using cell culture models. Choosing the appropriate culture well, or pre-treating the culture well surface to prevent adsorption of the compound of interest would be necessary to improve the recovery. Another possibility is that soyasapogenol B could be metabolized by Caco-2 cells during the incubation. However, this possibility does not seem likely as our TLC and HPLC analysis of the samples collected from the apical and basal chambers and the cell inserts did not reveal any compound which seemed to have structural characteristics similar to soyasapogenol B. Further investigation is needed to clarify these possibilities.

The apical-to-basolateral permeability coefficients of soyasaponin I and soyasapogenol B are summarized in Table 2. Our observations demonstrated that soyasaponin I was transported across the Caco-2 monolayer with a range of P_{trans} from 0.9 to 3.6×10^{-6} cm/sec at the three concentrations. After 4 h incubation, $2.9 \pm 1.2\%$, $0.9 \pm 0.4\%$, and $0.5 \pm 0.1\%$ of soyasaponin I, at 0.5, 1.0 and 3 mM, respectively, were received in the basal chambers. The calculated real permeability coefficients (P_{trans}) for soyasaponin I were similar to its apparent permeability coefficients (P_{app}), indicating the transport of soyasaponin I across the Caco-2 monolayer was much less than its simple diffusion across the polycarbonate membrane. Hence, the Caco-2 monolayer acted as a barrier against free

diffusion of soyasaponin I towards the basolateral side. Based on previous observations by Artursson and Karlsson (1991), examining the relationship between P_{app} values obtained from Caco-2 cell model and human *in vivo* oral absorption for a number of drugs, this range of permeability coefficients for soyasaponin I would imply some extent intestinal absorption in humans. For comparison, at 0.05 mM the apical-to-basolateral transcellular flux of glucose over Caco-2 monolayer had a P_{app} of 36.8×10^{-6} cm/sec, quecetin 5.8×10^{-6} cm/sec (Walgren *et al.* 1998), and genistein 20×10^{-6} cm/sec (Walle *et al.* 1999). The absorption of soyasaponin I might be less than those compounds in the human intestine. As indicated by $T_{0.1}$ in the Table 2, it would take 11.4 h to transport 10% of 0.5 mM soyasaponin I from the lumen to basolateral side of the gut.

The concentration-dependency of transport kinetics was examined as well in our study to characterize transport mechanisms. Our data showed that incubation up to 4 h gave a linear transport kinetics of soyasaponin I (Figure 1). The transport rate constants for soyasaponin I at different concentrations were not significantly different. The accumulated mass of soyasaponin I in the basal chambers was not significantly different after 4 h in spite of different concentrations in the apical chambers. This suggests that the apical-to-basolateral transcellular transport of soyasaponin I might be saturable and concentration-independent. We measured the accumulation of soyasaponin I in the cell monolayer as well to estimate the cellular uptake of soyasaponin I. The data are reported as the accumulated amount in nmoles/ μ g of protein, considering the variation in the numbers of cells on the inserts. As shown in Figure 2, the accumulation of 0.5 mM soyasaponin I in Caco-2 cells was lower than those of the two higher concentrations ($P < 0.01$), whereas it was not significantly different between 1 mM and 3 mM concentrations. It indicates that, like a

funnel-shaped transport mechanism, the apical-to-basolateral transport of soyasaponin I might be controlled at the basolateral membrane, and the uptake of soyasaponin I by Caco-2 cells could be saturable. In our study, the cell uptake of soyasaponin I at 0.5 mM was not saturated, but the output of soyasaponin I at the basolateral membrane had been saturated. At concentrations ≥ 1 mM, the cell uptake at the apical membrane and output of soyasaponin I at the basolateral membrane were all saturated. Therefore, it was observed that the accumulation of soyasaponin I in the cells became constant at the two higher concentrations, and the apical-to-basolateral transport rate of soyasaponin I was not different at the concentration range of 0.5 to 3 mM. A concentration-dependent transport rate may be observed if the concentrations of soyasaponin I used in the study were lower than saturation point of basolateral output. These findings suggest that transport of soyasaponin I by Caco-2 cells might involve a carrier-mediated mechanism. It is reasonable to infer that the absorption of soyasaponins in the intestine might be enhanced when the soyasaponin concentration increases within a very low range. The absorbed amount could be limited by the capacity of epithelial cells to uptake and transfer soyasaponins to basolateral side when high concentrations of soyasaponins are present in the gut.

Our observation showed that the permeability of soyasapogenol B was significantly lower than that of soyasaponin I ($P < 0.05$) regardless of the concentrations (Table 2). After 4 h incubation, only 0.2 to 0.8% of the total soyasapogenol B was transported to the basal chambers. Such a low apical-to-basolateral transport of soyasapogenol B was unexpected because we hypothesized that soyasapogenol B would be more absorbable since it is a smaller and more hydrophobic molecule compared to soyasaponin I. However, after adjusting the apparent permeability coefficients P_{app} with the permeability coefficients P_{filter}

for polycarbonate membranes in the absence of Caco-2 cells, we found that, unlike soyasaponin I, the real permeability coefficients P_{trans} of soyasapogenol B were significantly different from their apparent permeability and near zero. After carefully examining the diffusion kinetics of soyasapogenol B across the polycarbonate membrane filter, we found that only 0.1 to 0.4% of soyasapogenol B diffused through the membrane over 4 h. In contrast, about 60 to 96% of soyasaponin I passed through the membrane filter in the absence of Caco-2 cells. Therefore, the lack of absorption of soyasapogenol B in the Caco-2 cell model might not be a result of low transport by Caco-2 cells, but probably due to the low permeability through the polycarbonate membrane on which the cells grew. The collagenated polycarbonate membrane (0.45 μm) has been commonly used in transport studies and considered as a better supporting membrane for Caco-2 cell growth and differentiation with low adsorption to many drugs (Hidalgo *et al.* 1989; Artursson 1990). Some hydrophobic molecules, such as β -sitosterol and dexamethasone, have been shown to be permeable across the Caco-2 cell monolayer growing on the 0.45 μm polycarbonate membrane (Field *et al.* 1997; Artursson and Karlsson 1991). However, our observations suggested that hydrophobic molecules like soyasapogenol B might not be permeable to this membrane material. The very low absorbability obtained in the Caco-2 model studies might not represent the real absorbability of the compounds in this situation. Cogburn *et al.* (1991) demonstrated that the diffusion rate across the cell-free membrane filter of some lipophilic compounds, such as alprenolol and propranolol, was approximately half of that of a hydrophilic compound, mannitol. They indicated that different compounds could have very different permeability over the membrane which supported Caco-2 cell growth, and suggested a correction for transport across the blank membrane filter should be performed to

normalize the transport rates of all compounds relative to each other. In our study, it would have been better to assess the permeability of soyasapogenol B over different filter membranes first to choose the permeable membrane prior to transfer assay.

When examining the cell uptake of the test compounds, we found that 5.4 to 12.3% of soyasapogenol B was found in the cells. Unlike soyasaponin I, soyasapogenol B accumulated in the cells in a concentration-dependent manner (Figure 2). The uptake of soyasapogenol B was significantly higher than that of soyasaponin I at 0.5 and 3 mM concentrations ($P < 0.05$) but not different at 1 mM. The uptake of saponins or sapogenols by intestinal enterocytes is not known. However, the uptake of phytosterols by rat intestinal epithelial cells or Caco-2 cells has been reported. Field *et al.* (1997) reported the uptake of 0.1 mM sitosterol by Caco-2 cells was 0.5×10^{-3} pmol/insert after 4 h incubation, about half of the uptake of cholesterol. Compassi *et al.* (1997) demonstrated the uptake of sitosterol by Caco-2 cells was 25 to 75% at a concentration range of 4 to 100 μ M, and suggested that, in contrast to cholesterol absorption, the low absorbability of sitosterol was due to the low intracellular processing and basolateral secretion but not the low uptake at the brush border membrane (BBM). Our data indicates that soyasapogenol B could be uptaken by Caco-2 cells and accumulated in the cells. At the concentration range of 0.5 to 3 mM, the higher concentration of soyasapogenol B in the lumen, the more soyasapogenol B molecules could accumulate in the cells. Based on our observations from this study, we would not be able to conclude whether soyasapogenol B can be transported across the Caco-2 cell monolayer at this point, and the evidence is insufficient to predict absorbability of soyasapogenol B in the intestine.

Although the Caco-2 cell model has been widely used to evaluate absorbability and mucosal metabolism of many nutrients and phytochemicals, no study has reportedly used this model to measure saponin absorbability. In our study, the transport of soyasaponins was evaluated as the crystalline material of soyasaponin suspended in the aqueous transport buffer. However, the absorption of soyasaponins in the intestine might be more complicated because of the interaction of soyasaponins with other constituents of gut content. Some evidence indicated that the uptake and absorption of soy phytosterols could be facilitated by partition of bile salt micelles due to their structural similarity to cholesterol (Bhattaacharyya 1981; Ostlund *et al.* 2002). The uptake of sitosterol by BBM vesicle and Caco-2 cells appeared to be energy-independent and facilitated in a manner analogous to cholesterol uptake (Thurn-Hofer and Hauser 1990; Schulthess *et al.* 1996). It is likely that the absorption of dietary soyasaponins might be enhanced by partition of micelles in the intestine due to the amphiphilic nature of soyasaponins and their structural resemblance to cholesterol to some extent. The method for micelle incorporation with cholesterol or phytosterols has been well established and widely used in the cholesterol absorption related studies (Field *et al.* 1997; Compassi *et al.* 1995). Hence, future studies are warranted to modify the transport assay by incorporating soyasaponins into bile salt micelles and measuring the transport of soyasaponins in the Caco-2 cell model.

Lennernas (1997) reported the correlations of permeability from different *in vitro* transport models with human oral absorption data. It was found that permeability of the compounds via passive transport across the Caco-2 monolayer was comparable to those seen in the human intestine but was much lower for large hydrophobic compounds and carrier-mediated transported compounds. Since the transport kinetics of soyasaponin I across the

Caco-2 cell monolayer did not support a passive diffusion mechanism, it is reasonable to consider that a relatively higher absorption rate than that observed *in vitro* might be expected in the human intestine.

In order to determine if the concentrations of soyasaponin I and soyasapogenol B in our study damaged Caco-2 cells, acute cytotoxicity was estimated by measuring cell viability with dye exclusion test after the Caco-2 cell monolayer was treated with the same conditions used in the transport assay (Figure 3). The percent of dye-excluding cells ranged from 89.3 to 96.2% for all groups. Significant differences existed among the different treatment groups ($P < 0.01$). The percentage of viable dye-excluding cells in soyasaponin I treatment groups at all three concentrations was not significantly different from the control group. However, 1 mM and 3 mM of soyasapogenol B significant reduced percentage of viable dye-excluding cells in the culture compared to the control, while 0.5 mM soyasapogenol B did not show a cytotoxic effect.

It is not surprising that soyasaponin I did not display apparent toxic effect on the cells after 4 h of exposure. Sung *et al.* (1995) demonstrated that 1 h exposure of soyasaponins up to 600 ppm did not result in any change on the viability of human colon carcinoma (HCT-15) cells, whereas gypsophilla saponins decreased cell viability significantly at same concentrations. Berhow *et al.* (2000) reported that 50 to 250 $\mu\text{g/mL}$ soyasaponins had no acute cytotoxic effect on Chinese hamster ovary (CHO) cells. Our data further demonstrate that soyasaponins might not have an apparent cytotoxicity to intestinal epithelial cells even at concentrations up to 3 mM. It would be interesting to test whether soyasaponins are cytotoxic over a longer exposure period because of the approximate 24 to 48 h that food residue may stay in the gut and interact with gut epithelial cells. Although soyasaponins

appeared to be less cytotoxic compared to some other saponins, inhibition of carcinoma cells proliferation by soyasaponins has been reported (Sung *et al.* 1995; Lacaille-Dubois and Wagner 1996; Oh and Sung 2001). Therefore, the observed colon cancer inhibitory effect of dietary soyasaponin (Rao and Sung 1995; Koratkar and Rao 1997) might not be attributed to the direct cytotoxic activity but partially due to the growth inhibitory activity of soyasaponins.

Berhow's study (2000) showed that a 2 h treatment of 0.4 mM soyasapogenol B had no significant effect on CHO cell viability. Rowlands *et al.* (2002) reported a growth inhibitory effect of 10 μ M soyasapogenol B to human breast cancer cells. Our findings suggest that soyasapogenol B was cytotoxic to Caco-2 cells at concentrations above 1 mM. Soyasapogenol B showed protection of CHO cells against direct DNA damage induced by 2AAAF, possibly by intercepting reactive molecules inside the cells (Berhow *et al.* 2000). Our transport data also showed a high uptake of soyasapogenol B by Caco-2 cells. These evidences together suggest that, unlike soyasaponins interacting with the cell membrane, soyasapogenol B might exert its cytotoxicity inside the cells. Further research is required to explore the mechanisms and potency of cytotoxicity of soyasapogenol B on cancerous and normal cells.

In conclusion, our findings clearly show that ingested group B soyasaponins can be metabolized to soyasapogenol B by human intestinal microorganisms *in vivo* and excreted in the feces. The data generated in the Caco-2 transport experiment indicate that soyasaponins may have low absorbability in the human intestine. The apical-to-basolateral transport of soyasaponin I by the Caco-2 cells indicates that the absorption of soyasaponins may be limited by the capacity of epithelial cells to uptake and transfer soyasaponins to the

basolateral side in the gut. The uptake of soyasaponin I by gut epithelial cells might be saturable, in contrast, the uptake of soyasapogenol B might depend upon its concentration in the lumen. Dietary soyasaponins may not have an apparent cytotoxic effect on the gut enterocytes, while high concentration of their gut metabolites, soyasapogenols, might be relatively toxic.

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Legends to the figures:

Scheme 1. Structures of soyasaponin I and soyasapogenol B

Figure 1. The transport kinetics of soyasaponin I across Caco-2 monolayer at 0.5, 1, and 3 mM concentrations. The plot was the linear regression of the amount of soyasaponin I transported to the basal chamber versus incubation time. Each data point represents the mean \pm SD of three replicates.

Figure 2. The accumulation of soyasaponin I and soyasapogenol B at 0.5, 1, and 3 mM concentrations in the Caco-2 cells after 4 h incubation. The values are shown as mean \pm SD of duplicate. The letter a, b, and c indicate significant differences in the accumulations among the groups ($P < 0.05$).

Figure 3. The cytotoxicity of soyasaponin I and soyasapogenol B at 0.5, 1, and 3 mM concentrations to the Caco-2 cells. The cytotoxicity was presented as the percentage of viable cells harvested after treatment. The values are shown as mean \pm SD of duplicate. The letter a, b, and c indicate significant differences in cell viability among the groups ($P < 0.05$).

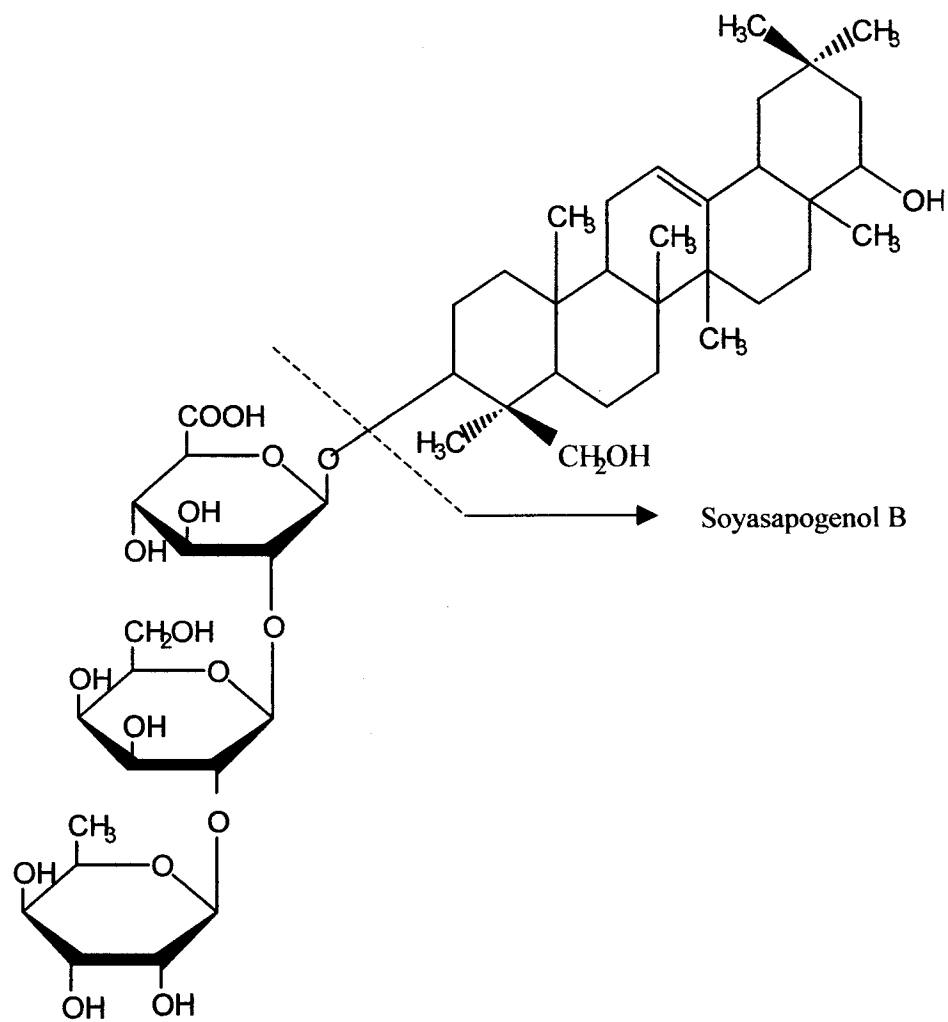
Scheme 1

Table 1. Soyasaponin I and soyasapogenol B contents in the urine and feces (N=8)

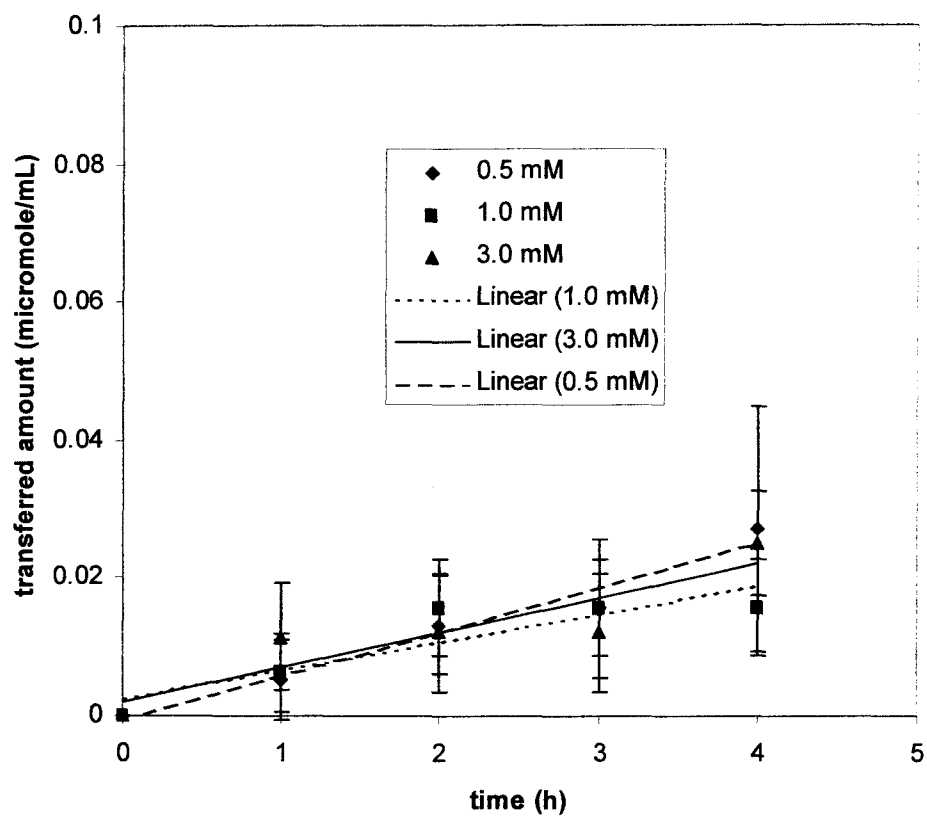
Sample		Soyasaponins (μ moles)	Soyasapogenol B (μ moles)
Urine	24 h	nd	nd
Feces	Day 0	nd	5.00 ± 3.54 (n = 3)
	Day 1	nd	11.65 ± 2.92 (n = 8)
	Day 2	nd	8.99 ± 1.15 (n = 6)
	Day 3	nd	5.24 ± 1.01 (n = 7)
	Day 4	nd	5.75 ± 0.68 (n = 4)
Total		nd	36.27 ± 3.59 (n = 8)

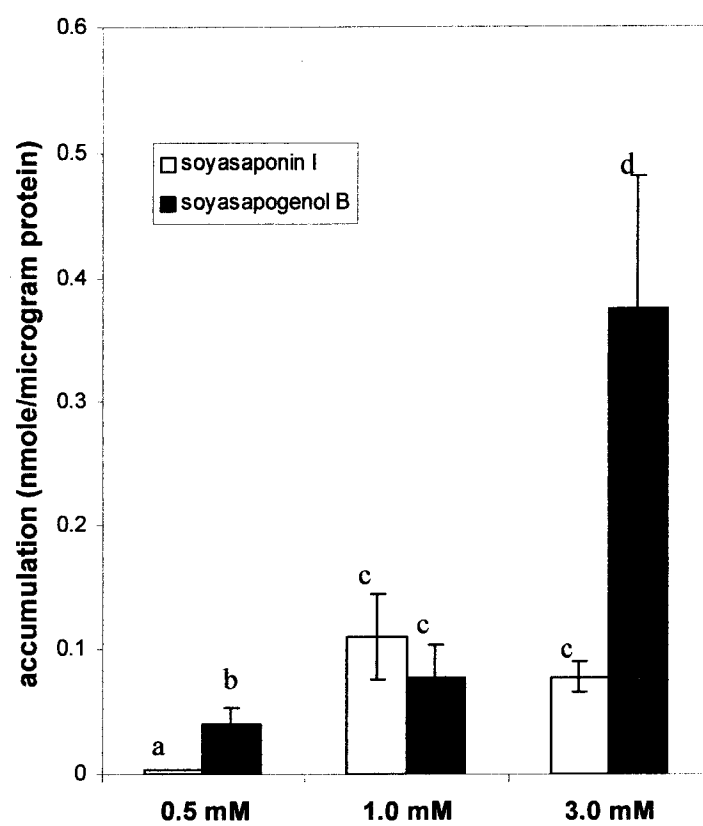
nd: not detected. The values are expressed as Mean \pm SE. Day 0 was the day the subjects ingested the single dose of soy extract. The dose was 435.6 μ moles/person.

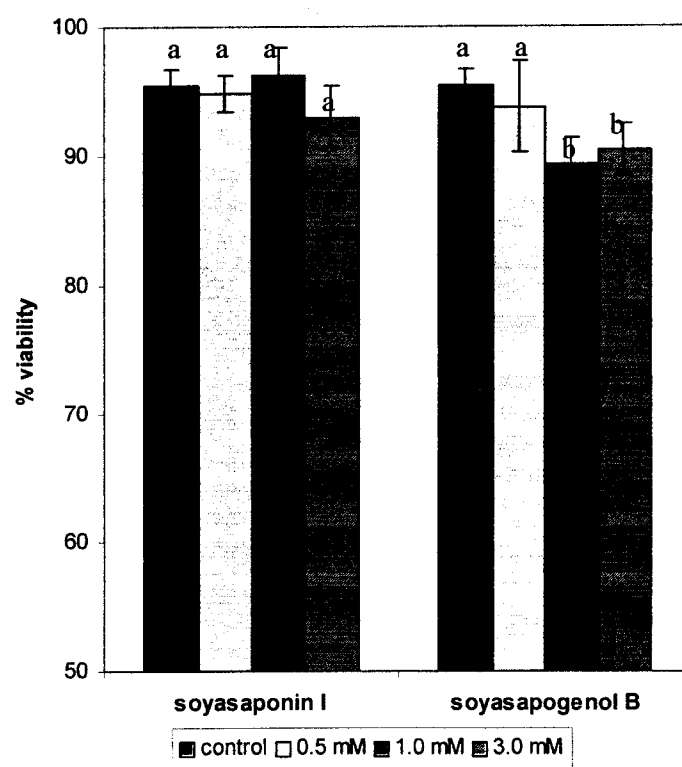
Table 2. The apical-to-basolateral transport of soyasaponin I and soyasapogenol B across the Caco-2 cell monolayer

Concentration (mM)	P _{app} (cm/sec)			P _{trans} (cm/sec)			T _{0.1} (hour)		
	0.5	1.0	3.0	0.5	1.0	3.0	0.5	1.0	3.0
Soyasaponin I	3.6±0.5×10 ⁻⁶	1.1±0.3×10 ⁻⁶	0.9±0.1×10 ⁻⁶	3.6±0.4×10 ⁻⁶	1.2±0.4×10 ⁻⁶	1.0±0.3×10 ⁻⁶	11.4	36.0	87.6
Soyasapogenol B	0.3±0.1×10 ⁻⁶	0.6±0.1×10 ⁻⁶	0.6±0.2×10 ⁻⁶	≤ 0	≤ 0	≤ 0	—	—	—

The real permeability coefficient P_{trans} was calculated from P_{app} and P_{filter}. T_{0.1} is the times needed to transfer 10% of the compounds from apical chamber to basolateral chamber and calculated from the respective transport rate constants based on P_{trans}.

**Figure 1**

**Figure 2**

**Figure 3**

GENERAL CONCLUSIONS

Soyasaponins, one of the main phytochemicals found in soybeans and soy products, have not attracted as much attention as soy isoflavones. A number of studies investigating soyasaponin's biological activities suggested that these compounds may have potential in improving human health. Most of these studies were conducted in *in vitro* systems and a few were animal experiments. The saponin doses used in these studies were not well justified and might be high relative to the possible physiological concentrations in humans. Therefore, the relevance of their findings to humans under *in vivo* conditions is not clear. In order to understand the mechanisms and predict the beneficial effects of dietary soyasaponins in humans, it is crucial to know the bioavailability and metabolism of these compounds. To our knowledge, our study will be the first to report the gut metabolism and absorption of dietary soyasaponins.

Our first study answered the question of what happens to soyasaponins in the human intestine after ingestion. The *in vitro* fecal incubation study revealed that ingested soyasaponins may be metabolized by human intestinal microflora present in the lower gut to molecules with fewer or no sugars attached to the aglycone. Individual variation exists in their ability to metabolize soyasaponins in the gut, probably due to the differences in gut microflora populations and/or bacterial enzyme activity. Further studies can be in the directions of identifying of the bacterial species in the human intestinal tract that hydrolyze soyasaponins, and exploring the factors that affect individual ability to metabolize soyasaponins in the gut.

Little was known about the bioavailability and absorption kinetics of soyasaponins in the human intestine. Our human feeding study further demonstrated the existence of gut microbial metabolism of soyasaponins *in vivo* by showing the presence of soyasaponin aglycones in the feces after oral dosing. However, our results from this feeding study could not provide a distinct conclusion on soyasaponin bioavailability since neither soyasaponins nor their gut metabolite, soyasapogenols, were detected in the urine of the subjects. Therefore, a Caco-2 transport assay was conducted to evaluate the absorbability of soyasaponins at the cellular level. The data generated in the Caco-2 transport experiment showed that ingested soyasaponins could be taken up by gut epithelial cells, but their absorption may be very low and limited by the capacity of epithelial cells to uptake and transfer soyasaponins to the basolateral side. In addition, the absence of direct cytotoxicity of soyasaponin I to the Caco-2 cells suggests that dietary soyasaponins may not be toxic to the gut enterocytes. In contrast, a high concentration of soyasaponin's gut metabolites, soyasapogenols, might be relatively toxic.

There were limitations in our studies. In the human feeding study, blood samples were not collected and analyzed because there were no established methods to analyze soyasaponins in the limited volume of blood samples. Our ability to identify the possible soyasaponin metabolites formed *in vivo* was also limited due to the complex nature of these compounds. It would be valuable for future work to have soyasaponins with an isotope-labeled aglycone to trace the fate of ingested soyasaponins in animal models or humans if allowed. In our Caco-2 transport experiment, the crystalline materials of soyasaponin I and soyasapogenol B were directly dissolved and applied in the apical chambers to test their absorbability. It is likely that the absorption of dietary soyasaponins might be enhanced by

partition of micelles in the intestine due to the amphiphilic nature of soyasaponins and their structural resemblance to cholesterol to some extent. Hence, future studies are warranted to modify the transport assay by incorporating soyasaponins into bile salt micelles and measuring the transport of soyasaponins in the Caco-2 cell model. The research on the mechanisms of soyasaponin transport by gut epithelial cells, and the mechanisms and potency of cytotoxicity and cytostatic effect of soyasapogenols on cancerous and normal cells, also deserves further exploration.

APPENDIX 1. FOOD LIST FOR HUMAN FEEDING STUDIES

AVOID the following foods which may contain soy soyaaponins during the wash-out days and experiment period.

I. Soy Protein Isolate

Soybeans
Tofu
Soymilk, Soymilk powder
Soybean sprouts
Tempeh
Miso soup
Special K frozen waffles
Carnation Instant Breakfast-chocolate malt flavored

II. Food Containing Texturized Vegetable Protein

Frozen pizza
Burritos
Morningstar Farm breakfast links, patties, strips
La Choy lobster egg rolls
Liquid non-dairy creamers

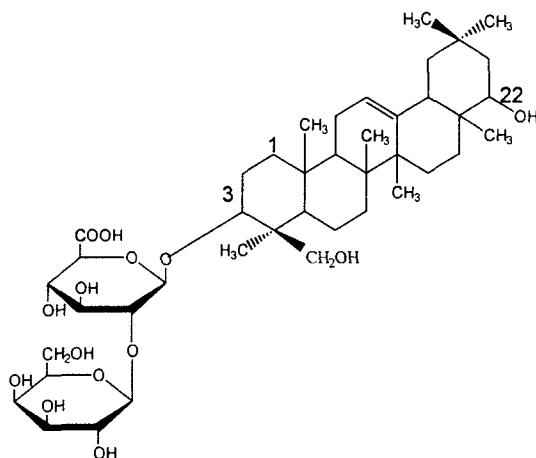
III. Foods Containing Hydrolyzed Vegetable Protein (HVP)

Most chip dips (French onion and some others)
Garden vegetable flavored cheese spreads
Tombstone frozen pizza with meat
Some franks (*John Morrel*, *Hormel* Light & Lean, etc.)
Sauce mixes - gravy (usually brown), chili, etc.
Knorr soup mixes
Knorr dry sauce mixes except "Pesto"
Canned soups (usually those containing vegetable & meat, like chicken and mushroom)
La Choy foods as well as oriental style mixes, etc. (containing HVP or soy sauce)
Soy sauce has soybeans pr protein extracts from soybeans
Ramen noodles containing HVP and /or soy sauce powder
Heinz worstershire sauce (containing HVP in soy sauce)
Hiland Red Hot Piplets (most other chips and snacks were fine)
Uncle Ben's Rice mix
Herbal magic salad dressing, Girad's salad dressing
Most bacon flavored bits

IV. Food Rich in Soyaaponins

Green beans, kidney beans, broad beans, mung beans,
Chickpeas, garden peas, green peas, pea flour,
Ginseng and ginseng tea, lupin seeds, lentils, alfalfa sprouts
Herbal medicines and teas

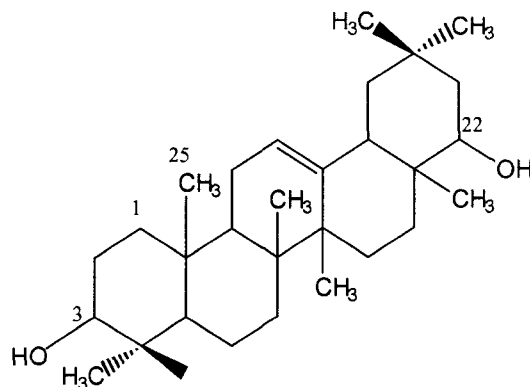
APPENDIX 2. ^{13}C AND ^1H -NMR SPECTRA DATA FOR SOYASAPONIN III



Carbon position	Chemical shifts ^a	
	^{13}C	^1H
Aglycone moiety		
3	89.2	3.42s
12	121.1	5.19s
13	142.9	
22	80.7	3.34d (6.8)
24	62.8	3.3d (12.0), 4.05d (11.9)
3-O-glucuronopyranosyl		
1'	103.5	4.34d (7.7)
2'	78.9	
3'	74.4	
4'	71.8	
5'	76.9	
6'	172.0	
2'-O-galactopyranosyl		
1''	103.6	4.49d (7.3)
2''	71.9	
3''	73.2	
4''	68.8	
5''	75.2	
6''	59.8	

*: ^{13}C APT-NMR and ^1H -NMR spectra were acquired on a Varian VXR-300 spectrometer. The sample was dissolved in DMSO- d_6 . The ppm values are relative to the chemical shift of TMS. The numbers in the parentheses are J values.

APPENDIX 3. ^{13}C AND ^1H -NMR SPECTRA DATA FOR SOYASAPOGENOL B



Position	Chemical shifts ^a		Position	Chemical shifts ^a	
	^{13}C	^1H		^{13}C	^1H
1	38.5		16	27.5	
2	25.6		17	36.4	
3	76.3	3.493s	18	44.8	2.088s
4	42.0		19	46.2	
5	55.8		20	30.5	
6	18.7		21	41.3	
7	33.0		22	81.0	3.44t (5.1)
8	39.7		23	22.5	
9	47.8		24	64.7	3.425d (11.1), 4.20d (10.8)
10	37.2		25	16.2	
11	23.6		26	16.0	
12	122.0	5.246s	27	25.5	
13	143.9		28	27.9	
14	42.8		29	32.5	
15	28.0		30	19.9	

*: ^{13}C APT-NMR and ^1H -NMR spectra were acquired on a Varian VXR-300 spectrometer. The sample was dissolved in chloroform- d_6 . The ppm values are relative to the chemical shift of TMS. The numbers in the parentheses are J values.

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